JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2008. Vol. **2**(1), p. 01-10

Alkaline Polysaccharidases Produced in Solid State Cultures by Alkalophilic Fungi Isolated from Argentina

N.L. Rojas[1], S.F. Cavalitto[1-2], M. Cabello[3], R.A. Hours[1] and C.E. Voget[1]*

[1]Research and Development Center for Industrial Fermentation (CINDEFI). CONICET-UNLP. School of Science, La Plata National University. 47 y 115, (B1900ASH) La Plata, Argentina.
[2]Department of Science and Technology, Quilmes National University. Roque Saenz Pena 352, (B1876BXD) Bernal, Argentina.
[3]Institute Spegazzini, La Plata National University, 53 # 477, (1900) La Plata, Argentina.

(Received: 20 November 2007; accepted: 26 December 2007)

Alkalophilic fungi (*Acremonium implicatum*, *Acremonium sp.*, *Acrostalagmus luteo-albus*, *Beauveria bassiana*, *Clonostachys rosea*, *Doratomyces stemonitis*, *Paecilomyces lilacinus* and *Volutella ciliata*) isolated from forest alkaline soils (pH 9 to 10) of the Eastern plain in Buenos Aires province (Argentina) were grown in solid-state cultures (SSC) and subjected to a screening of alkaline polysaccharides. Specific growth rates (μ) in agar media with different combinations of glucose or sucrose (as carbon and energy sources, CES) and tryptone or peptone (as N-sources) were determined. All fungi showed comparable growth behaviours in the different media demonstrating that they show similar nutritional requirements. These fungi were able to grow in SSC (pH 9.0–9.5) using perlite as inert support impregnate with liquid media containing glucose, pectin or V-8® juice as CES. *A. luteo-albus* and *C. rosea* displayed the highest capacity to metabolize pectin as well the greatest pectin lyase activity. Alkaline pectate lyase activity was produced by all fungi (particularly by *A. luteo-albus*) in almost all media. Alkaline RGase activity was produced by *A. implicatum*, *A. luteo-albus* and *C. rosea*. All fungi showed alkaline cellulase (*B. bassiana* and particularly *A. luteo-albus*) and xylanase (*Acremonium sp.*, *A. luteo-albus*, *D. stemonitis* and mainly *A. implicatum*) activities. *A. luteo-albus* produced the richest enzymatic pool and may be a promising source of alkaline enzymes.

Keywords: Alkalophilic fungi, Alkaline enzymes, Plant cell-wall degrading enzymes, *Acrostalagmus luteo-albus*.

Biotechnology advances during the last decades, mainly in Genetics and Protein Engineering, have assigned a fundamental role to enzymes within many industrial processes[1]. Nowadays, there is an increasing trend to replace traditional chemical processes by biotechnological techniques involving microorganisms and/or enzymes. They not only constitute a less aggressive alternative for the environment but also are more economically viable[2]. Enzymes must be effective under working conditions of the industrial processes. Therefore, the screening of high number of microorganisms is the first step of getting a very efficient biocatalyst under the experimental physicochemical condition.

The biotechnological potential of fungal depolymerizing enzymes as likely biological catalysts in a variety of industrial processes has

*To whom all correspondence should be addressed.
Tel/fax: +54 221 483-3794;
E-mail.: voget@biotec.org.ar

drawn attention from researchers worldwide. There is an extensive bibliography about acidic or neutral fungal depolymerases, routinely used in the food, textile and other industries[3]. On the contrary, relatively few reports are available about fungal alkaline depolymerases and their use in industrial bioprocesses is a new niche of biotechnological research. Among them, alkaline proteases, cellulases, xylanases, pectinases and mannanases show the greatest present and potential applications as tools for biotechnological exploitation [4].

Microfungi from alkaline soils have been examined by Rai *et al*.[5] and Nagai *et al*.[6,7] and those from soils with high amounts of calcium carbonate were studied by Vardavakis[8]. In addition, a group of rhizospheric soil microfungi was isolated from the mesophilic (undisturbed and disturbed) tala (*Celtis tala*) and coronillo (*Scutia buxifolia*) forest located in the coastal region of Buenos Aires province in Eastern central Argentina by Cabello and Arambarri[9]. This region presents characteristic alkaline soils due to its high calcium carbonate content. These fungi, belonging to the Fungi Imperfecti, have been taxonomically identified and preliminary characterized. They have been classified according to their ability to grow under different pH values, ranging from alkali-tolerant (they grow from pH 6 to 10) to alkalophilic (which could grow at pH 10, but not at pH 5-6)[10]. Due to the extreme environmental conditions where these fungi naturally grow, it is reasonable to suppose they could be able to produce enzymes active at high pH values. Of particular interest in this connection would be those fungi that produce a broad spectrum of polysaccharide-degrading alkaline enzymes.

In this work, we are reporting the fungal growth of 8 indigenous strains in different alkaline media. The strains were screened as sources of alkaline enzyme activities in solid-state cultures (SSC) using perlite impregnated with different alkaline media. Particularly, production of some alkaline depolymerising enzyme activities such as pectinases (polygalacturonase, pectin and pectate lyase and rhamnogalacturonase), cellulases and hemicellulases (xylanase) was evaluated.

MATERIAL AND METHODS

Chemicals and Inert Support Materials

Citrus pectin (degree of esterification (DE) 53 and 90%), polygalacturonic acid sodium salt (PGA), beechwood xylan (Xyl), larchwood arabinogalactan (AG) and CM-cellulose sodium salt were obtained from Sigma Chemicals (St. Louis, MO, USA). Soybean rhamnogalacturonan (RG) was purchased from Megazyme (Ireland). V-8® juice (Campbell Soup Company) was obtained from a local market and clarified by the addition of $CaCO_3$ and centrifugation[11]. Potato dextrose agar (PDA) and tryptone were from Merck. Soy peptone was from Oxoid. Malt extract (Britania, Argentina) agar (MEA) contained malt extract 20 g, agar 15 g and distilled water 1000 ml. All other chemicals used were commercially available and of analytical grade.

Agricultural perlite (PERLOME®) was a gift of Laboratorios Biagro SA (Argentina). Perlite was sieved into different particle size fractions. The fraction +80/-100 mesh was used as inert support for SSC. The apparent density of the material was 0.16 g/cm^3 and the water holding capacity was 7 ml/g of dried inert support. For the determination of the desorption isotherm, perlite was autoclaved for 30 min at 121ºC with excess water. Then, the excess of water was drained off, and 2.5 g-portions were incubated in sealed plastic containers where the relative humidity was adjusted between 90 and 100% with glycerol solutions [12].

Microorganisms and inoculum preparation

Different fungal strains (*Acremonium implicatum* (Gilman & Abbot) Gams LPS cult # 764, *Acremonium sp.*, *Acrostalagmus luteo-albus* (Link: Fr) Zare, Gams et Schroers LPS cult # 748, *Beauveria bassiana* (Bals.) Vuill. LPS cult # 803, *Clonostachys rosea* (Link: Fr.) Schoers LPS cult # 930, *Doratomyces stemonitis* (Pers. ex Steud.) Morton & G. Sm. LPS cult # 984, *Paecilomyces lilacinus* (Thom) Samson LPS cult # 792, and *Volutella ciliata* Alb. & Schw. ex Fr. LPS cult # 766) were used for this study. They have been previously isolated from calcium carbonate rich soils as described by Cabello and Arambarri[9] and are deposited at the Institute Spegazzini fungal culture collection (La Plata

National University, Argentina). Stock cultures were maintained on MEA (*A. implicatum*, *Acremonium sp.*, *B. bassiana*, *C. rosea* and *P. lilacinus*) or PDA (*A. luteo-albus*, *D. stemonitis* and *V. ciliata*) tubes under a layer of mineral oil at 4ºC. Inocula were prepared by streaking a loop of the stock cultures on PDA or MEA dishes and incubation for 7 days at 30ºC. Mycelia plugs (5 × 5 mm) were obtained from the margin of young colonies. Conidia were harvested after 10 days of incubation by flooding the agar dish with 10 ml of 0.05% Tween 80 sterile solution and gently scraping the surface with a sterile spatula. Concentration of the conidia suspensions was determined with a Neubauer chamber.

Media and culture conditions

A reference medium (RM), proposed by Contreras Esquivel *et al.*[13], was used with some modifications. The composition of RM was: carbon and energy source (CES) 5.0 g, N-source 2.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, citric acid 0.01 g, buffer solution ($K_2HPO_4 \cdot 2H_2O$ 0.9 g/l; Na_2CO_3 1.0 g/l) 100 ml and distilled water 900 ml. When required, agar was added to a final concentration of 15 g/l. Three stock solutions of these medium components (CES, buffer solution and the rest of components) were separately sterilized by autoclaving. Final pH values of the reconstituted media ranged from 9.0 to 9.5.

Growth studies with different CES and N-sources were performed on 9 cm dishes containing 20 ml of agarized RM. The following four combinations of CES and N-sources were tested: glucose/tryptone, sucrose/tryptone, glucose/soy peptone and sucrose/soy peptone. Inoculation was performed with one agar plug placed upside down at the centre of the dish. Dishes were incubated under controlled humidity and temperature conditions (100% humidity, 30°C). Growth was evaluated by measuring the colony diameter (in mm) after 120, 168, 240 and 269 h. A linear model proposed by Pirt[14] for colony growth and adapted by Trinci[15] for filamentous fungi was used to estimate the specific growth rate (μ). Tests were conducted in triplicate for each fungus, and average (± SD) was calculated for all fungi on each CES and N-source combination.

SSC using perlite were carried out in Petri dishes containing 2.5 g of inert support, which was sterilized for 30 min at 121ºC and then impregnated with 15 ml of RM containing tryptone and different CES already inoculated with conidia at a final concentration of 10^6 conidia/ml. The CES used were: glucose (Glu medium, reference), pectin DE 60% (Pec medium) and clarified V-8 juice (V-8 medium) diluted to 3 g/l of total sugars (final concentration). Dishes were incubated for 72 h under controlled humidity and temperature conditions (100% humidity, 30°C).

Sample treatment in SSC

The whole content of each dish was homogenized manually with a glass rod and weighted. Culture extracts were obtained by stirring 5 g of culture in 10 ml of distilled water for 30 min at room temperature with a magnetic rod. After measuring the pH, the suspension was filtered through Whatman # 40 filter paper. The filtrate was freeze-dried and kept at 4ºC. When required, the freeze-dried sample was suspended with enough amount of deionized water in order to give a 10 × concentrated solution, as comparing to the original extract. The solution was centrifuged in an Eppendorf microfuge to eliminate residual solids and kept refrigerated until used. The remaining solid medium was dried at 70ºC for water content determination.

Enzyme activity assays

Pectinase, polygalacturonase (PGase), rhamnogalacturonase (RGase), arabinogalactanase, cellulase and xylanase activities were determined with 2.0 g/l substrate (citrus pectin DE 90%, PGA, RG, AG, CM-cellulose and Xyl, resp.) solutions in Tris-HCl buffer (50 mM, pH 9.0), by measuring the formation of reducing groups by the Somogyi-Nelson method (16). Pectin and pectate lyase activities were assayed spectrophotometrically at 235 nm (ε_{235} = 4600 $M^{-1}cm^{-1}$) using 20 g/l citrus pectin (DE 90) or PGA, respectively, in Tris-HCl buffer (50 mM, pH 9.0) (17). All enzyme activities were determined at 37ºC. One unit of activity was defined as the amount of enzyme which releases 1 μmol of reducing sugars or insatured products per minute under the above mentioned reaction conditions.

Analytical determinations

Carbohydrate analysis in culture samples was carried out as follows. Glucose was measured

with a glucose oxidase/peroxidase enzymatic kit (Wiener, Argentina)[18]. Pectin was estimated as galacturonic acid (GALA) by the *m*-hydroxydiphenyl-H_2SO_4 method for determination of uronic acids[19] and total sugars in V-8 medium were determined by the phenol/H_2SO_4 technique[20] using glucose as standard.

Soluble sugars (glucose, fructose and sucrose) in clarified V-8 juice were determined by HPLC. A Waters HPLC modular system equipped with a pump (model 1525), RI detector (model 2414) and a Shodex SC1011 column, was used for analysis. The injection volume was 10 µl. Samples were eluted with HPLC grade water at a flow rate of 1.0 ml/min at 85°C. Glucose, fructose and sucrose were identified and quantified by comparison of their retention times with those of standards[21]. Soluble pectin in clarified V-8 juice was precipitated with 3 vols. of cold 0.05 N HCl in ethanol. The mixture was kept for 60 min in an ice-water bath and centrifuged (5,000 × *g*, 4°C) for 10 min. The pellet was then washed with cold 0.05 N HCl in ethanol and dissolved in water. Pectin content was determined as previously mentioned[5].

RESULTS AND DISCUSSION

Fungal growth in agar media

A preliminary characterization of growth in different agar media using the selected fungi was carried out to determine the most proper medium composition for further use in SSC. Fungal strains were grown in RM containing four different combinations of CES and N-sources. Colony growth was evaluated by using the linear model of Trinci (r = 0.95-0.99). Analysis of µ values (average ± SD) for all strains was performed for each medium combination. In all cases, no substantial differences in µ values (revealed by the comparative low SD) of the 8 strains in the same medium were found. Therefore, the average µ of all strains in each medium was calculated in order to compare fungal growth of different tested media (Table 1). From these results it was assumed that all fungi have similar nutritional requirements. The combination of glucose and tryptone (Glu/Try) was chosen as a new reference medium for further SSC studies because it seems to have a slight advantage as compared to the others.

Table 1. Specific growth rates (for all the fungi tested) determined in the different combinations of CE- and N-sources

Medium		Specific growth rate
CE-source	N-source	Average ± SD (h^{-1})
Glu	Try	0.0068 ± 0.0013
Suc	Try	0.0064 ± 0.0016
Glu	Pep	0.0064 ± 0.0013
Suc	Pep	0.0063 ± 0.0016

Glu, glucose; Try, tryptone; Suc, sucrose; Pep: peptone.

Fungal growth in SSC using impregnated perlite

SSC usually approximates natural conditions for fungal growth and enzyme production[22]. SSC using perlite as inert material impregnated with the Glu medium (reference), Pec medium and V-8 medium, were chosen as experimental culture condition to simulate the natural alkaline environment of the fungi. Raw perlite is a generic name which designates certain type of siliceous rock. Expanded perlite is obtained by heating up to 800ºC (by evaporation of the bounded water) which increases its volume between 4 and 20 times respect to the original one. The resulting material is white, with high porosity, neutral pH and able to absorb several times its weight in water. The use of expanded perlite as inert material in SSC has been recommended because it allows the design and use of adequate testing media (all concentrations of the components are known) and facilitates the extra-cellular product extraction[23].

A critical aspect of SSC is the control of water activity (a_W) because most water is absorbed into the porous solid material. Reduction of a_W to values lower than the optimum affects fungal growth resulting in a longer lag phase and lower values of µ and biomass production. Although fungi can tolerate $a_W \cong 0.95$, here it was assumed a $a_W \cong 0.98$ as the minimum value in order to

avoid adverse effects on fungal growth and enzyme production. In order to have a_W = 0.98, the perlite water content should be 6.0 g/g as revealed by the desorption isotherm.

Table 2 shows the culture pH (initial and final), final water content and remaining CES levels after three days of cultivation on perlite impregnated with 3 media (Glu, Pec and V-8). Perlite initially contained about 6.3 g of water per gram of dry support, and culture pH was about 9.3. In all cultures, final water content was approximately 6.0 g of water per gram of dry support ($a_W \geq 0.98$), and final pH was about 9.0. These results are demonstrating that the chosen fermentation conditions were adequate for the control of these culture parameters during fungal growth.

All the studied fungi showed the capability to efficiently grow in glucose, measured as consumption of CES (about 100% for all the fungi). On the other hand, only two fungi (*A. luteo-albus* and *C. rosea*) showed high capacity to assimilate pectin (CES consumption was above 95%); whereas pectin consumption was partial (@ 50 – 60%) in the cultures of *A. implicatum*, *D. stemonitis*, *P. lilacinus* and *V. ciliata* and low (about 40%) in *Acremonium sp.* and *B. bassiana*. Analysis of soluble carbohydrates in diluted

Table 2. Cultivation of alkalophilic fungi in SSC under alkaline conditions using perlite as inert material impregnated with different media.

Fungus	Medium	Culture pH		Final water	Final CES
		Initial	Final	content (g/g)	(mg/l)
A. implicatum	Glu	9.14	8.97	6.09	ND
	Pec	9.11	8.91	6.04	2397
	V-8	9.17	9.01	6.06	271
Acremonium sp.	Glu	9.23	8.85	5.99	ND
	Pec	9.44	8.74	6.01	3054
	V-8	9.26	8.90	6.02	207
A. luteo-albus	Glu	9.19	9.04	5.99	ND
	Pec	9.28	8.99	6.07	226
	V-8	9.35	9.08	5.99	197
B. bassiana	Glu	9.31	8.93	5.99	ND
	Pec	9.52	9.12	6.04	2767
	V-8	9.30	9.03	6.05	410
C. rosea	Glu	9.31	8.94	6.06	ND
	Pec	9.52	8.96	6.06	151
	V-8	9.30	9.09	6.05	328
D. stemonitis	Glu	9.14	8.83	5.99	ND
	Pec	9.11	8.73	5.97	2155
	V-8	9.17	8.95	6.03	284
P. lilacinus	Glu	9.23	9.19	6.02	ND
	Pec	9.44	9.30	6.07	1915
	V-8	9.26	9.27	6.02	268
V. ciliata	Glu	9.19	8.84	6.04	ND
	Pec	9.28	9.03	6.05	2229
	V-8	9.35	8.98	5.97	1069

Initial CES (carbon and energy source) concentrations: glucose (Glu medium) 5 g/l, pectin (Pec medium) 5 g/l and total carbohydrates (V-8 medium) ≅ 3.0 g/l. Initial humidity: ≅ 6.3 g of water per gram of dry material.
See text for experimental details. ND: not detected.

clarified V-8 juice revealed the presence of glucose (1.44 g/l), fructose (1.51 g/l) and sucrose (0.05 g/l) in addition to pectic substances (0.15 g/l). Sugar utilization in V-8 cultures was about 90% indicating that most of these fungi (with an exception of *V. ciliata*) easily assimilate the simple sugars present in V-8 juice. This result is consistent with the growth of the fungi in Glu medium. *V. ciliata* seems be able to assimilate some but not all of the CES (except from glucose) present in V-8 medium.

Enzyme activities in SSC using impregnated perlite

Screening of alkaline enzyme activities in fungal cultures was carried out using pectic (pectin, PGA and RG) and non-pectic substrates (CM-cellulose and Xyl). Table 3 shows the alkaline enzymatic activities recovered from the SSC using different media (Glu, Pec and V-8). The presence of enzyme activities able to degrade highly esterified pectin (DE about 90%) was observed mainly in all the cultures of *A. luteo-albus*. Pectin lyase activity was remarkable high in some cases: *C. rosea* in V-8 medium, *D. stemonitis* in V-8 medium and *P. lilacinus* in Pec medium. When comparing the pectin lyase activity values with those of pectinase, it can be assumed that pectin lyase activity might be the

Table 3. Alkaline enzyme activities (mU/ml) on pectic and non-pectic substrates detected in SSC using perlite impregnated with different culture media.

Extract		Pectic substrate					Non-pectic substrate	
		Pectin (DE 90%)		PGA		RG	CM-cellulose	Xylan
Fungus	Medium	Pectinase	Pectin lyase	PGase	Pectate lyase	RGase	Cellulase	Xylanse
A. implicatum	Glu	5.6	0.74	1.5	0.49	8.4	5.4	18.9
	Pec	ND	3.76	ND	3.19	ND	1.3	26.1
	V-8	ND	5.48	ND	10.55	ND	0.3	29.2
Acremonium sp.	Glu	1.1	0.98	ND	0.41	ND	0.2	1.0
	Pec	1.7	ND	ND	7.28	2.3	8.8	15.7
	V-8	4.5	7.94	ND	0.82	ND	3.5	14.7
A. luteo-albus	Glu	11.0	22.90	8.2	1.23	6.0	31.7	12.5
	Pec	12.5	38.45	10.3	16.50	10.0	42.0	10.4
	V-8	10.1	49.10	8.7	5.65	7.3	35.4	2.6
B. bassiana	Glu	1.2	0.74	ND	1.80	ND	3.3	0.8
	Pec	1.7	1.88	ND	6.87	4.0	ND	2.9
	V-8	ND	1.96	1.4	7.69	1.5	12.3	3.4
C. rosea	Glu	2.0	1.31	1.6	ND	ND	8.1	8.7
	Pec	ND	4.83	ND	1.31	ND	1.8	9.4
	V-8	ND	18.25	ND	1.55	12.3	3.1	0.1
D. stemonitis	Glu	ND	ND	ND	1.23	ND	0.5	1.9
	Pec	ND	ND	ND	9.82	ND	0.2	10.0
	V-8	1.6	12.93	ND	5.15	ND	2.7	11.4
P. lilacinus	Glu	3.4	ND	ND	ND	1.5	4.3	8.9
	Pec	1.4	16.69	ND	2.78	ND	ND	0.3
	V-8	2.9	0.82	ND	1.88	ND	3.7	6.8
V. ciliata	Glu	2.4	0.90	1.2	1.15	ND	ND	ND
	Pec	1.4	2.70	7.0	3.52	ND	2.9	1.9
	V-8	1.6	4.91	ND	2.70	ND	3.8	2.0

See text for experimental details. ND: not detected.

principal responsible for the degradation of pectin by most of the fungi tested. It should be noted that pectinase activity is regarded here as the combination of different enzyme activities resulting in the degradation of pectin (determined by measuring the formation of reducing groups) irrespectively of the involved mechanism. In some cases, pectinase activity was detected although pectin lyase was low (*A. implicatum*, *C. rosea* and *V. ciliata* in Glu medium) or null (*P. lilacinus* in Glu medium). These results (relatively high pectinase activity with low or null pectin lyase activity) might be explained by the potential presence of pectinesterase (EC 3.1.1.11), an enzyme activity which was not tested in this research because it is out of the scope of the present work. Pectinesterase de-esterifies methoxylated pectin releasing free carboxyl groups (and methanol) and thus decreasing the degree of esterification of the substrate. Consequently, activities of pectate lyase and/or PGase could contribute significantly to the pectinase activity.

These results are in agreement with the capability of these filamentous fungi to use pectin as CES. *A. luteo-albus* and *C. rosea* display the greatest pectin degrading activity as well as the highest capacity to grow on pectin as the only CES. The comparatively high PGase activity found in all *A. luteo-albus* cultures suggests the presence of PGase activity in its pectolytic pool irrespectively of the medium composition used; therefore, it seems to be a constitutive enzyme in this fungus.

Pectin and pectate lyase activities were lower in almost all fungi in Glu medium compared with Pec or V-8 media. Similar results were found in PGase activity except for *A. implicatum*, *A. luteo-albus* and *C. rosea*. In addition, enzyme activities detected in Glu medium are, in general, lower than those found in Pec or V-8 media. Glucose repression in these fungi probably could explain this effect. Simple sugars such as glucose, arabinose and galactose have been reported to suppress the synthesis of alkaline pectinases, possibly due to catabolite repression[24]. Nevertheless, the situation is more complex in V-8 medium due to the presence of a mixture of different CES, some of them possibly either involved in Glu derepression of Glu-repressed genes or acting as enzyme synthesis inducers. It is known that induction of pectinases involves constitutive low level expression of at least some structural genes. Basal amounts of pectinase activity could produce trace amounts of degradation products that are supposedly chemically modified, resulting in potent inducers in clarified V-8 juice containing pectin (or partially degraded fragments). The substantially high values of pectin lyase activity in *Acremonium sp*., *C. rosea* and *D. stemonitis* in V-8 medium in comparison with those found in Glu and Pec media could be related to the above mentioned induction mechanism.

Alkaline pectate lyase activity was produced by all fungi (particularly by *A. luteo-albus*) in almost all media. In general, Glu medium yielded lower activities in comparison with V-8 and particularly Pec media. Pectin seems to be the best inducer for this enzyme. The presence of pectate lyase activity is very interesting because this enzyme is mainly produced by phytopathogenic fungi (in addition to various groups of bacteria including actinomycetes) but not by saprotroph alkalinophilic fungi like those studied here. Until recently, it was thought that pectate lyases were secreted mainly by plant pathogens, their action resulting in the maceration of plant tissues. Pectate lyase action in phytopathogens results not only in plant cell-wall degradation, but also in the activation of defence systems, presumably through the release of oligogalacturonides from the plant cell wall, which then function as defense elicitors. In the case of saprotroph alkalinophilic fungi, alkaline pectate lyase could contribute to the decomposition of plant deposits in soil by breaking down pectin polymers for nutritional purposes.

PGase, pectin and pectate lyase are pectolytic enzymes acting on the homogalacturonan or smooth region of the pectin molecule. An attempt was made to detect the RG main-chain degrading activities by using a commercial soybean RG preparation as substrate. RG is part of the hairy regions in pectin-polymers, a major component of the plant cell walls. The degradation of the backbone of the hairy regions is performed by enzymes designated rhamnogalacturonases (RGases). RGase attacks the bonds between galacturonic acid and

rhamnose residues in the RG region of pectin. RGase activity is regarded here as an enzyme activity resulting in the degradation of RG irrespectively of the hydrolytic or trans-eliminative mechanisms. The presence of an alkaline RGase activity in the cultures of *A. implicatum* in Glu medium, *A. luteo-albus* in all media and *C. rosea* in V-8 medium is remarkable high. It seems that the induction-repression mechanisms of RGases in these 3 fungi are quite different.

Amounts of RGase are limited in commercial enzyme preparations and, to our knowledge, the enzyme activities reported are restricted to acidic and/or neutral environments. RGase II from *A. aculeatus* exhibits optimum activity in the pH range of 3-4[25], which is lower than the pH of most plant materials and lower than desired pH in most industrial processes. RGase from *Trametes sanguinea* was stated to be used at pH 5.0[26]. Nevertheless, it is important to provide a variety of different RGases (in respect to mode of action, pH and temperature range) in order to be able to exploit the desirable actions of RGases under widely varying technical process conditions, especially for the industries dealing with modifications of plant cell walls for e.g. human nutrition and for animal feed (e.g. liquefaction of fruits, vegetables, cereals, oil fruits and seeds). In particular there is a need for RGases which are active at a high pHs. The above mentioned alkaline RGase activities would be the first fungal enzyme activities reported till now which degrade RG at pH 9.0.

Table 3 also shows the alkaline enzyme activity values detected on non-pectic polymeric substrates such as CM-cellulose and xylan, which represent the remaining main carbohydrate components of the plant cell wall. Unlike the case of pectinolytic enzymes, all these filamentous fungi present the capability to degrade CM-cellulose and xylan. It is interesting to emphasize the alkaline cellulolytic activity produced by *B. bassiana* in V-8 medium and particularly *A. luteo-albus* in all media. Also, the presence of high alkaline xylanolytic activities of *Acremonium sp.* and *D. stemonitis* detected in both Pec and V-8 media, *A. luteo-albus* in Glu and Pec media, and mainly *A. implicatum* in all media. In general, most of these fungi, with exception of *A. luteo-albus*, yielded pectolytic activities comparatively low or null. Different induction-repression patterns could be assumed. It seems that alkaline cellulase activity is under catabolite repression by glucose in *Acremonium sp.* and *V. ciliata* but not in the cases of *A. implicatum* and *C. rosea*. In addition, alkaline xylanase activity was strongly repressed by glucose in *Acremonium sp.*, *B. bassiana* and *D. stemonitis*.

CONCLUSIONS

Our results indicate that SSC using perlite impregnated with different media is an adequate culture system to simulate natural environments to study extra-cellular enzyme production. In addition, it could be stated that the studied fungi can be used as alternative sources of alkaline enzyme activities with potential industrial applications. In particular, *A. luteo-albus* is the most interested fungus because it has demonstrated to have a high versatility concerning growth and extra-cellular alkaline enzyme activity production. It displays the richest alkaline pectin degrading enzyme activities pool of the fungi tested, additionally to alkaline cellulase and xylanase activities. This fungus also presents the ability to degrade arabinogalactan under alkaline conditions (data not shown). In other words, the fungus is also able to depolymerize lateral ramifications of pectin hairy regions in addition to smooth regions. This is a very notable outcome considering its low biodiversity contribution index [9]. From these results we can conclude that this filamentous fungus is highly adapted to degrade the plant cell walls in alkaline environments. Molecular characterization of this fungus in order to reconfirm its identity is in progress. Future studies on *A. luteo-albus* enzyme pool should be devoted to the isolation and characterization of the enzymes responsible of the activities detected.

Work on the utilization of alkaline pectinases remains underdeveloped as only a few reports are available on applications of these enzymes. Therefore, biotechnological potential applications of *A. luteo-albus* pectinases, particularly enzymatic retting of bast fibers from jute, flax, hemp, or ramie for the manufacturing of textiles is considered.

ACKNOWLEDGMENTS

These studies were supported by Grants for Scientific Research from UNLP (11/X417) and CONICET (PIP # 5931).

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