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Induction of Extracellular Lytic Enzymes from *Aureobasidium pullulans*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The objective of this work was to analyze the production of some extracellular lytic enzymes of the fungus *Aureobasidium pullulans*.

Methodology: The fungus was isolated from Valencian orange and was cultivated on Mathur medium modified with polygalacturonic acid (2% w/v) or xylan (2% w/v) as a carbon source, and they were incubated at 28°C, with constant stirring at 100 rpm, and at different times, the supernatant was harvested by filtration, and the extracellular lytic activity was determined by the Nelson method modified by Somogy, as well as the extracellular protein by the Lowry method.

Results: The production of extracellular polygalacturonase and xylanase was induced, finding that the former has an optimal induction time at 7 days, with glutamic acid as a nitrogen source, and is

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stable at 4°C and 28°C and an optimal temperature and activity times of 60-80°C and 4 hours, while xylanase presents an optimal induction time of 9 days, with glutamic acid as nitrogen source, and very stable at 4 and 28°C, with optimal temperature and time of activity of 28°C and 8 hours.

Conclusion: The fungus exhibits both polygalacturonase and xylanase activity. The polygalacturonase activity has a maximum induction time at 7 days, while xylanase has an optimal induction time at 9 days of incubation at 28°C. The xylanase activity was very stable at 4°C and 28°C, as it retains an activity of 100% at both temperatures after five days of incubation, while polygalacturonase retains 82.5% of its initial activity at 4°C, and 56.7% at 28°C.

Keywords: Induction; lytic enzymes; activity; Aureobasidium pullulans.

1. INTRODUCTION

Plant pathogenic fungi produce different extracellular enzymes capable of degrading the components of plant cell walls. It has been postulated that lytic enzymes are involved in a variety of processes such as: penetration, growth, and development of the fungus, and to understand the pathogenesis, it is important to establish the function of different lytic enzymes such as pectinases, cellulases, polygalacturonases, xylanases, laccases, etc., since it has been suggested that these are the first enzymes that are expressed when the fungus infects the plant [1,2,3], and this expression, as well as the production and degradation of polymers of the fungus depend on the culture conditions, such as pH, aeration, and nitrogen source [1,3,4]. In the literature, there are many studies that try to establish the role of extracellular lytic enzymes in the pathogenicity of fungi such as the induction of extracellular cellulases in the phytopathogenic fungus *Colletotrichum lindemuthianum* [1], the induction of lytic enzymes during the interaction of *Ustilago maydis* with corn [5], the production of cellulases by *Aureobasidium pullulans* LB83 [6], the carboxymethyl cellulase production by *Trichoderma reesei* [7], the production of pectinases of industrial interest in yeast species isolated from soils and fruits of the Valle del Cauca region, Colombia [8], the isolate of fungi from natural compost and produce cellulases in submerged fermentation [9], the production of cell-wall degrading enzymes, such as cellulase and pectinase by *A. pullulans* NAC8 through induction using orange peels [10], and endo-xylanases of *U. maydis* Involved in fungal filamentation [11].

The citrus diseases caused by fungi cause economic losses worldwide, and in some cases, they reach up to 50% of production total of the fruits [12], and the application of fungicides reduces the losses significantly, but these are still between 5-10% of total production [13]. A significant number of the diseases that affect citrus fruits are caused by fungi, these diseases are divided into three groups, depending on where the infection occurs. Thus, on the one hand, there are diseases produced by soil fungi, those of the aerial part and those that cause post-harvest damage [12]. Among the invasive fungi, the only one that attacks citrus is *Armillaria mellea*, and practically the rest of the fungi in the soil belong to the group of saprophytic fungi (*Fusarium, Phythopthora,* and *Verticillium*) that, depending on the environmental conditions, can parasitize large number of plants, including citrus [14], other examples are: white root rot by *Rosellinia necatrix* [15], gummies, root neck and trunk base rot, and absorbent root rot by *Phythophthora nicotiane* and *P. citrophthora* [16], the basal death of citrus by *Ceratocystis fimbriata* [17], the pink disease by *Corticium salmonicolor* [18], the drying of branches by *Lasiodiplodia theobromae* [19], the leaf spot by *Alternaria tenuissima* [20], vascular wilt on Asparagus by *Fusarium oxysporum* [21].

On the other hand, the yeast-like fungus *A. pullulans* is an imperfect fungus, which is common in nature and typically found growing in soil and water, as well as on weathered wood and many other plants, is a saprophytic fungus with a worldwide distribution, and it is more frequently found in the soil, leaves and wood of trees. It's also common to isolate it from kitchens and bathrooms, and it can also damage painted walls [22]. Its growth temperature varies from 2 to 35°C with an optimum of 25°C. *Aureobasidium* allergy has frequently been described among atopic patients, but its actual significance remains uncertain. It seems to be the cause of some cases of asthma [23]. It is considered a skin and nail saprobe, and cases of onychomycosis, keratitis, peritonitis, and even invasive infections have been reported in immunocompromised patients, and catheter-related septicemia due to this fungus [23, 24], and this fungus has been recognized as a potential producer of several enzymes including laccases [25], pullulan [26], cellulase [27], esterase [28], pectinases [29], Xylanases [3], and polygalacturonases [30]. Additionally, *A. pullulans* can secrete xylanases in the extracellular medium containing agricultural by-products [3, 26, and 29], which leads to costs reduction in enzyme production, and it has been suggested that polygalacturonases participate in penetration of the host by the degradation of the layer of pectin, and this induction is only detected during host plant infection in some fungi [2], while xylanases are a group of enzymes with carbohydrase activity, specifically classified as glycosidase, that hydrolyze xylan polysaccharide to xylose, and are common in bacteria and fungi that degrade plant matter. These organisms will be used to produce the enzyme that will be used in the manufacture of feed, and too are involved in fungal filamentation and proliferation on and inside plants [11]. Therefore, the objective of this work was to analyze the production of some extracellular lytic enzymes of the fungus *A. pullulans* isolated from *Citrus sinensis*.

2. MATERIALS AND METHODS

2.1 Sample

From the Republic market in the City of San Luis Potosí, a batch of 100 pieces of Valencian Orange was sampled, from which 10 pieces were taken at random.

2.2 Development of the Microorganism

The 10 pieces of Valencian orange were placed in plastic bags separately, to favor the conditions of humidity and heat for the development of contaminants for a period of 10 days at 28°C, to carry out the sampling.

2.3 Sampling

From each one of the oranges a scraping of the affected part of the peel was taken and it was inoculated in medium Potato Dextrose Agar (PDA) by the chopping technique, incubating at 28°C for 7 days. Also, a sample of the juice of each of the oranges was taken by aspiration, it was emptied in PDA medium at 28°C for a period of 7 days.

2.4 Isolation of the Fungus

The resulting colonies were seeded independently in the PDA medium by the sting technique, and were incubated for 7 days, at 28°C, until obtaining pure colonies of the developed fungi.

2.5 Identification of the Fungi

The identification of the fungal colonies was carried out based on their macro and microscopic characteristics. The macroscopic study was done with the naked eye, observing the morphology, color, and growth form of the colonies. The microscopic study was carried out using the transparent adhesive tape technique on the surface of the colony and subsequently gluing it, by pressure, on a slide on which a drop of 1% methylene blue (w/v) was previously placed (Fig. 1 A and B). At first it has a yeast-like appearance, white or pink in color, which over time darkens and acquires a folded appearance, the hyphae are short, multisept, thick-walled, and pigmented. It produces small spicules from which hyaline conidia are produced, which are formed at the same time at different points in the conidiogenous cell. Subsequently, the conidia undergo a budding process to form chains of other conidia that become pigmented [23].

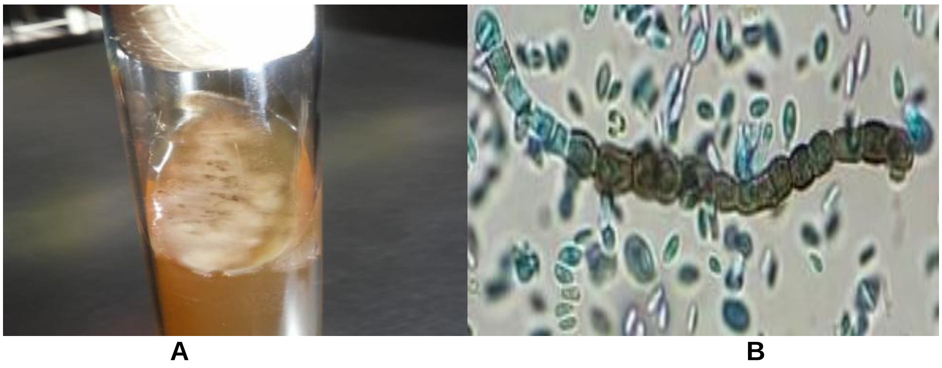

Fig. 1. Macroscopic (A) and microscopic (B) characteristics of the fungus *Aureobasidium pullulans* isolated from contaminated oranges.

2.6 Fungus Culture

The fungal isolate was routinely kept in Potato Dextrose Agar (PDA) at 28°C. For its propagation, 200 mL Erlenmeyer flasks containing 100 mL of modified Mathur's medium ($MgSO_4.7H_2O$, 2.5 g; KH_2PO_4, 2.72 g) were used, bringing to 1 L with distilled water, final pH 5.5 [31].The media were supplemented with different carbon sources: polygalacturonic acid (2% w/v), for polygalacturonase and xylan activity (2% w/v), for xylanase, the already prepared flasks were inoculated with a roast of a colony of 5 days of growth of *A. pullulans* incubating at 28°C, with constant shaking (100 rpm).

2.7 Determination of Protein

This was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard [32].

2.8 Determination of Extracellular Lytic Activities

To measure the enzymatic activities of the fungus under different conditions, whether of xylanase and/or polygalacturonase, the reducing sugars of the polysaccharide hydrolysate were determined by the Somogy method modified by Nelson [33], as follows: for the activity polygalacturonase was used as a substrate polygalacturonic acid (previously washed with deionized water and dried at 80°C/2 h) at 0.3% (p/v) in 50 mM acetate regulator pH=5.30, added 7.5 mM EDTA; for xylanase, xylan (previously washed with deionized water and dried at 80°C/2 h) at 1% (p/v) in 50 mM acetate buffer pH=5.00; and expressing the activity as nanograms of galacturonic acid or xylose released per minute. Specific activity was calculated for 1 mg of protein (Fig. 2). All the experiments were carried out at least 2 times in duplicate.

3. RESULTS AND DISCUSSION

3.1 Fungus Growth, Induction Kinetics and Stability of Extracellular Lytic Activities[a]

The production of extracellular lytic enzymes was induced, as described in the methodology, finding only polygalacturonase and xylanase activities, using polygalacturonic acid and xylan as the sole carbon source, respectively. Furthermore, no extracellular lytic activity was induced when glucose was used as the sole carbon source (Table No. 1). The polygalacturonase activity has a maximum induction time at 7 days, while that of xylanase has an optimal induction time at 9 days of incubation at 28°C (Figs. 3 and 4), in addition, the xylanase activity was very stable at 0°C, 4°C and 28°C, since at 7 days of incubation, there is an activity of 100%, 98% and 95%, respectively, while polygalacturonase retains 73%, 71% and 43.1% of its initial activity (Figs. 5 and 6). With respect to the polygalacturonase, has been reported extracellular enzymatic activities and physiological profiles of *A. pullulans* colonizing fruit trees with 1.25% commercial citrus pectin as substrate [34], from Brazilian semi-arid environments [35], the production of transfructosylating enzymes using low-cost sugarcane molasses by *A. pullulans* FRR 5284 [36], and the time of induction is different to the 10 days of incubation, reported for polygalacturonase of the same species of fungus, although isolated from the waters of the Danube river (Italy), and great stability to 40°C [37], 2 days for the same activity from *Bacillus licheniformis* KIBE-IB3 using agro waste pectin [38], 4 days for a endo-polygalacturonase from *Fusarium proliferatum* isolated from agro-industrial waste and pectin how substate [39], for two Polygalacturonases isolated from the digestive juice of the snail *Limicolaria flammea* [40], an exo-polygalacturonase of *A. pullulans* isolated from Saharan oil of Algeria grown on tomato pomace, and crude enzyme provided 30% and 60% of clarification of citrus and apple juices, also shows that the amount of reducing sugars released after enzyme treatment in the apple and citrus juice was highly increased, exhibiting 100% residual activity after 1 h at 60°C [30]. For xylanase, it was reported an optimum time of 24 h for *A. pullulans* CCT 1261, using rice bran as xylan source, [3], 4 days of incubation for the xylanase activity of laboratory type strain [41], 2-3 days for xylanase from *A. pullulans* in submerged cultivation [42], 86.1% and 90.2% of xylooligosaccharides production by crude and partially purified xylanase from *A. pullulans* after 1 day of incubation, and great stability to 40°C [43], crude xylanase produced with *A. pullulans* NRRL Y–2311–1 from wheat bran [44], 2 days for a endo-xylanase from *Ustilago maydis* [11], 3 days for xylanase of bacteria of the genus *Ruminococcus* with xylan 1% (p/v) how substrate [45], and 4 days for xylanase production by *Penicillium echinulatum* in submerged media containing cellulose amended with sorbitol [46], and in *Streptomyces* sp. RCK-SC, It retains about 82% of its activity at 70 °C after 2 hours of incubation and it is stable over the pH range 8-10 [47].

3.2 Effect of Different Nitrogen Sources on Growth, Enzyme Production and Protein Secretion

The polygalacturonase and xylanase activities were markedly affected by the nitrogen source. As observed in Table 2, the substitution of glutamate by other organic nitrogen substrates such as ammonium chloride, asparagine, urea, and glutamine did not induce activity. Other nitrogen sources such as ammonium nitrate reduced the activity by 90%, compared to that seen with glutamate. The maximum growth of the fungus was obtained with glutamic acid, followed by glutamine and asparagine, and no correlation was observed between growth and protein secretion. With respect to xylanase, ammonium chloride and glutamic acid induced the highest xylanolytic activity, followed by ammonium nitrate and urea (42.4 and 38.2 of specific activity, respectively), but the maximum growth was obtained with glutamic acid as nitrogen source (382 mg dry weight) followed by asparagine (48.6 mg) and glutamine (40.1 mg). Regarding glutamate, a correlation was observed between growth and protein secretion (Table 2). An exo-polygalacturonase of *A. pullulans* isolated from Saharan oil of Algeria grown on tomato pomace using yeast extract (as nitrogen and vitamin source) does not influence in the polygalacturonase activity [30], for the production of transfructosylating enzymes by *A. pullulans* FRR 5284, sodium nitrate was a most effective nitrogen source [48], yeast extract added with sodium nitrate was the better nitrogen source for the production of the extracellular enzymatic activity in *B. licheniformis* KIBE-IB3 [38], the enzyme production of *T. viride* (BITRS-1001) was more efficient in media containing casein and peptone as nitrogen sources [46]. In *Streptomyces* sp. RCK-SC, it was found that urea (0.25% w/v) is a good inductor of the activity [48], and for polygalacturonase activity by *Bacillus sphaericus* (MTCC 7542), casein hydrolysate and yeast extract used together as organic nitrogen source gave best results, and ammonium chloride was found to be the most suitable inorganic nitrogen source [49].

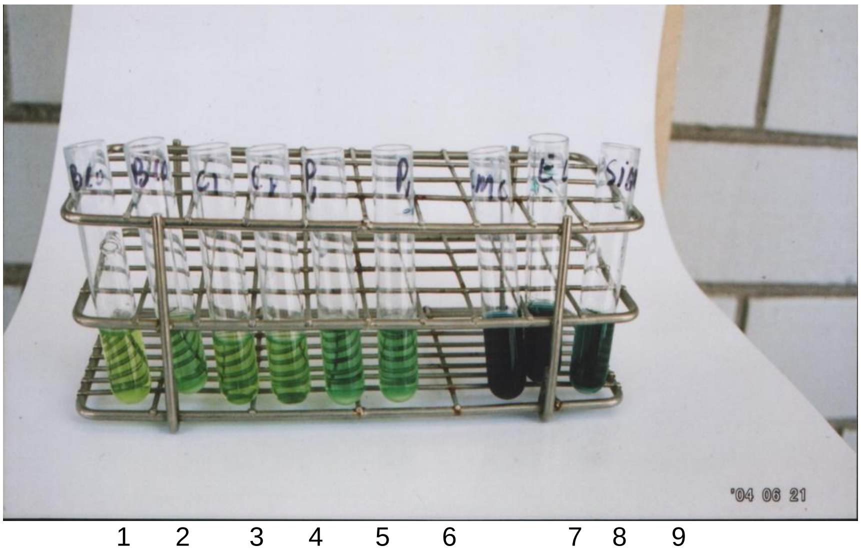

Fig. 2. Nelson-Somogy test for the determination of reducing sugars.

1, 2.- Whites

3, 4.- Negative controls

5, 6.- Problems

7.- Positive control using cellulase from Trichoderma reesei ATCC26921 (Sigma Chemical Co.) with carboxymethylcellulose as substrate.

8.- Positive control using cellulase from T. reesei ATCC26921 (Sigma Chemical Co.) with ethylcellulose as substrate.

Table 1. Induction of extracellular lytic enzymes by *Aureobasidium pullulans*

Substratum	Dry weight (mg)	Extracellular protein (mg/mL)	Specific activity[b] (Reducing sugars)
Polygalacturonic acid	278	190	Polygalacturonase16.44
Xilan	390	76	Xylanase 26.31
Glucose	1276	137	Polygalacturonase 0.00 Xylanase 0.00

a.- 7 days of incubation at 28°C, 100 rpm
b.- Polygalacturonase: milligrams of galacturonic acid/min/mg of protein
Xylanase: milligrams of xylose/min/mg of protein.

Table 2. Growth of *A. pullulans* in different sources of nitrogen and induction of the activities of extracellular polygalacturonase and xylanase[a]

a. Polygalacturonase

Nitrogen source[b]	Growth (mg of dry weight)	Extracellular protein(µg/mL)	Polygalacturonase activity[c]
Asparagin	75.1	24.4	0.00
Urea	13.2	24.4	0.00
Glutamine	76.1	36.8	0.00
Ammonium chloride	409.0	5.1	0.00
Ammonium nitrate	38.7	38.7	0.00
Glutamic acid	94.7	24.4	16.4

b.- Xilanase

Nitrogen source[b]	Growth (mg of dry weight)	Extracellular protein(µg/mL)	Polygalacturonase activity[c]
Asparagin	48.6	85.7	13.6
Urea	24.2	48.0	38.2
Glutamine	40.1	69.2	34.7
Ammonium chloride	32.0	5.1	99.72
Ammonium nitrate	30.7	44.8	42.41
Glutamic acid	382.0	44.0	94.6

a.- 7 and 9 days of incubation at 28°C, 100 rpm, with polygalacturonic acid or xylan as a carbon source. pH=5.5, respectively.
b.- all nitrogen sources were added at a concentration of 5.28 g/L.
c.- As milligrams of galacturonic acid/min/mg of protein.
d.- As milligrams of xylose/min/mg of protein.

With respect to xylanase activity, for the endoxylanase and β-xylosidase activities by *Aspergillus awamori* in solid-state fermentation, were higher when sodium nitrate was used as the nitrogen source, when compared with peptone, urea, and ammonium sulfate [50], too, ammonium sulphate was the most appropriate inorganic nitrogen source for xylanase production and urea increased xylanase activity slightly by *Trichoderma harzianum* 1073 D3 [51], on the production of xylanase by mutant strain of *Aspergillus niger* GCBMX-45, was observed when ammonium sulphate (0.2%) was found to be best nitrogen source for optimum enzyme production [52], for thermostable alkaline xylanase from *Anoxybacillus kamchatkensis* NASTPD13, among all the tested inorganic and organic nitrogen sources, ammonium sulfate, yeast extract, and peptone were found best for growth and xylanase production [53], for the activity in *B. subtilis*, the better inductor was ammonium sulphate [54], and by the xylanase roduction by *Streptomyces costaricanus* 45I-3, both source of nitrogen i.e., nitrogen ammonium sulphate and yeast extract were able to produce xylanase 8 days after incubation [55].

3.3 Effect of Initial pH on Growth and Induction of Extracellular Lytic Activities

Both, the growth, and the production of polygalacturonase and xylanase by the fungus exhibited an optimal initial pH of 5.5; Although the growth (in dry weight) was higher than the other pH's analyzed for both enzymes, the enzymatic activities detected at these values were lower, compared to the value obtained at pH 5.5 (Table 3). About, an exo-polygalacturonase of *A. pullulans* isolated from Saharan oil of Algeria grown on tomato pomace has an optimum pH of 5.0 and was stable at a broad pH range (5.0–10) [30], a polygalacturonase of the same species of fungus, although isolated from the waters of the Danube river (Italy) with the pH optimum 4.6 [36], a pH of 7.0 for the enzyme of from *B. licheniformis* KIBE-IB3 using agro waste pectin [38], a pH of 3.6 for an endo-polygalacturonase from *F. proliferatum* [39], a pH of 5.99 for the polygalacturonase from *T. viride* (BITRS-1001) [47], in *Streptomyces* sp. RCK-SC, it was found that he enzyme exhibits optimum activity at pH 10 [49], and for polygalacturonase activity by *B. sphaericus* (MTCC 7542), the optimal pH for bacterial growth and polygalacturonase production was 6.8 [50]. With respect to extracellular xylanase, it was reported an optimum pH of 6.0 for *A. pullulans* CCT 1261 [30], a pH of 5.0 for xylanase from *A. pullulans* in submerged cultivation [41], by crude and partially purified xylanase from *A. pullulans* after 24 h of incubation [3], crude xylanase produced with *A. pullulans* NRRL Y–2311–1 [44], the enzyme activity of xylanase of *Anoxybacillus kamchatkensis* was found to be similar between pH 7 and 9, with maximum activity at pH 9, and retained 71–100% of its maximal activity [51], an optimum pH of 7.0 for the activity by *B. subtilis* [52], and the xylanase production by *L. meleagris* KKU-C1 isolated from compost at pulp and paper industry, exhibited the highest activity at pH 8.0, and crude xylanase retained over 52% of the original activity in the pH range of 7.0-11.0, respectively, after incubation at 4°C for 60 min, and crude xylanase retained over 69% of the original activity up to 50°C [56], and an optimum pH of 6.0 of xylanase of *Pichia pastoris* [57].

3.4 Effect of Incubation Temperature on the Enzymatic Activities of Polygalacturonase and Xylanases of the Fungus *A. pullulans*.

Aliquots of 200 µL (20 µg of protein) of the extracellular lytic activities were incubated for 60 minutes under the conditions described above in a final volume of 1.7 mL at temperatures: 28, 37, 45, 50, 60 and 80^{o}C; A dependence of lytic activities with respect to temperature was observed, the maximum activity being detected at 60^{o}C and 80^{o}C for polygalacturonase and at 28^{o}C for xylanase, respectively (Figure 7). Has been reported in the literature, an exo-polygalacturonase of *A. pullulans* isolated from Saharan oil of Algeria has an optimum temperature of 60^{o}C and showed stability over a range of temperature (5–90^{o}C) [30], for a polygalacturonase of the same species of fungus, although isolated from the waters of the Danube river (Italy) the optimum temperature was 50^{o}C [37], 37°C for the enzyme of *B. licheniformis* KIBE-IB3 [38], 43.4°C for an endo-polygalacturonase from *F. proliferatum* [39], the activity of both polygalacturonases from the snail *L. flammea* was decreases with increasing heating time (5-120 min) and temperature (50-70°C) [40], in *Streptomyces* sp. RCK-SC, it was found that the enzyme exhibits optimum activity at temperature between 60 and 70°C [48], and for polygalacturonase activity by *B. sphaericus* (MTCC 7542), the optimal the temperature for bacterial growth and polygalacturonase production was 30^{o}C [49]. With respect to extracellular xylanase, it was reported an optimum temperature of 40°C for *A. pullulans* CCT 1261 [3], 50°C by crude and partially purified xylanase from *A. pullulans* after 24 h of incubation [43], 40°C for crude xylanase produced with *A. pullulans* NRRL Y–2311–1 from wheat bran [44] and *B. subtilis* a biobleaching agent [54], for *S. costaricanus* 45I-3, the optimum temperature was 28°C [55], and the xylanase production by *Leucoagaricus meleagris* KKU-C1 isolated from compost at pulp and paper industry, and an xylanase of *P. pastoris* exhibited the highest activity at 50°C [55, 57].

3.5 Effect of Protein Concentration on the Enzymatic Activities of Polygalacturonase and Xylanases of the Fungus *A. pullulans*

The activity of the extracellular lytic enzymes was determined as described in material and methods, using variable amounts of protein, and incubating for 60 minutes at 28^{o}C. The results (Figure 8) showed that the enzyme activity was directly proportional to the enzyme concentration up to 600 µg/mL for polygalacturonase and 400 µg/mL for xylanase activity. In the literature was report a concentration of 25 mg/mL of protein by crude and partially purified xylanase from *A. pullulans* after 24 h of incubation [3], an enzyme concentration of 240 U/g of xilooligosacharydes from crude xylanase produced with *A. pullulans* NRRL Y–2311–1 from wheat bran [44], 2.73

IU/mL of endoxylanas is reported for an endo-1, 4-β-xylanase production by *Aureobasidium pullulans* using agro-industrial residues [57], 244.02 U/mL for a cellulase-free xylanase from *Trichoderma inhamatum* [58], 14.5, 1.6 mg/mL of a *T. inhamatum* strain cultivated in liquid medium with oat spelts xylan [59], and 2.8 mg/mL from the fungus *Penicilium citrinum* was successfully synthesized and expressed in the yeast *P. pastoris* [60].

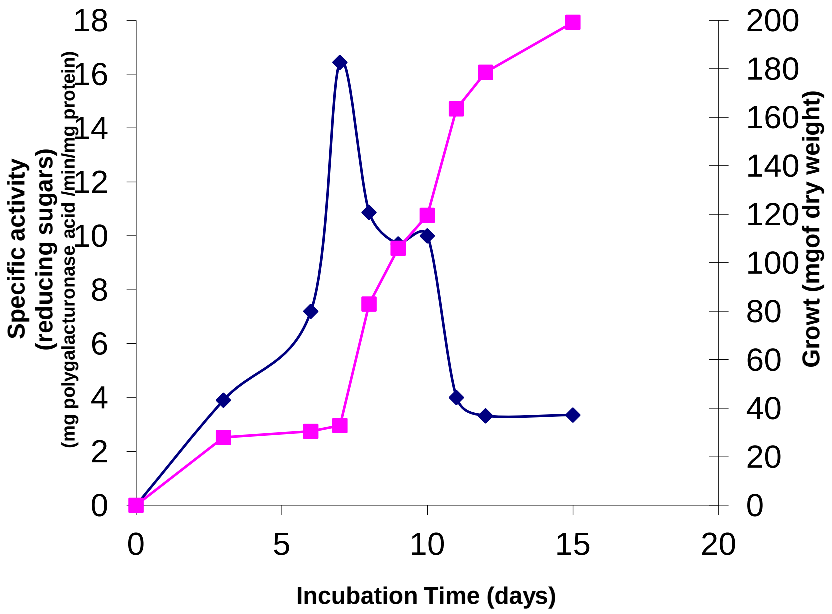

Fig. 3. Kinetics of activity induction of extracellular polygalacturonase of *A. pullulans.* Mathur medium 2.0% (w/v) polygalacturonic acid. 28°C, 100 rpm

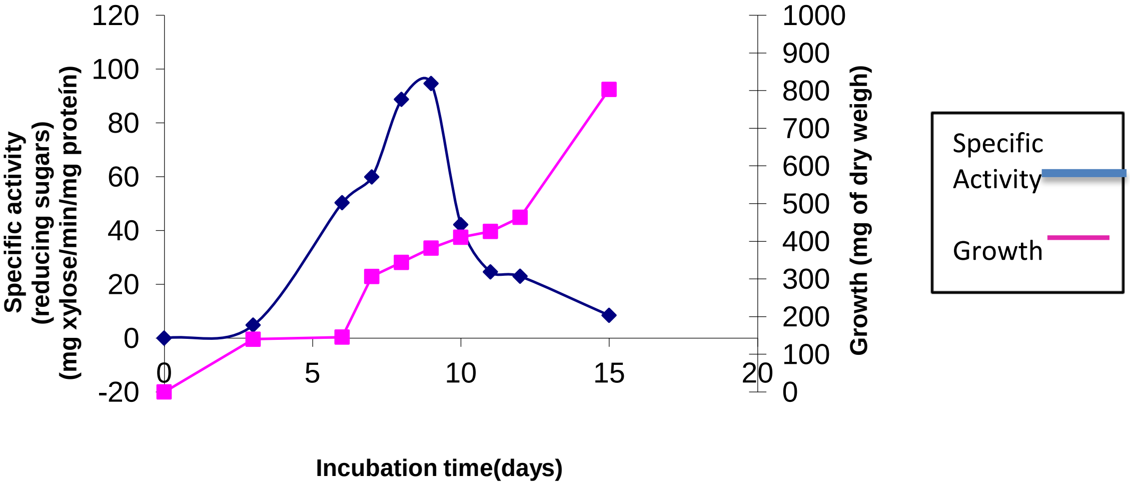

Fig. 4. Kinetics of activity induction of extracellular xylanase of *A. pullulans.* Mathur medium 2.0% (w/v) xylan. 28°C, 100 rpm

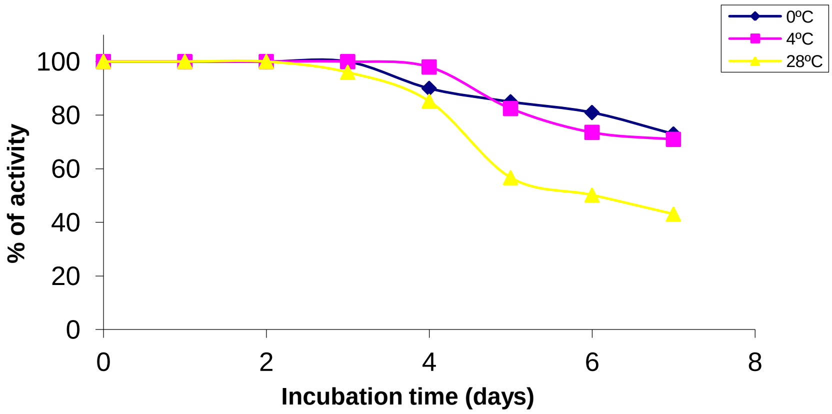

Fig. 5. Stability of the extracellular polygalacturonase of *A. pullulans* at different temperatures

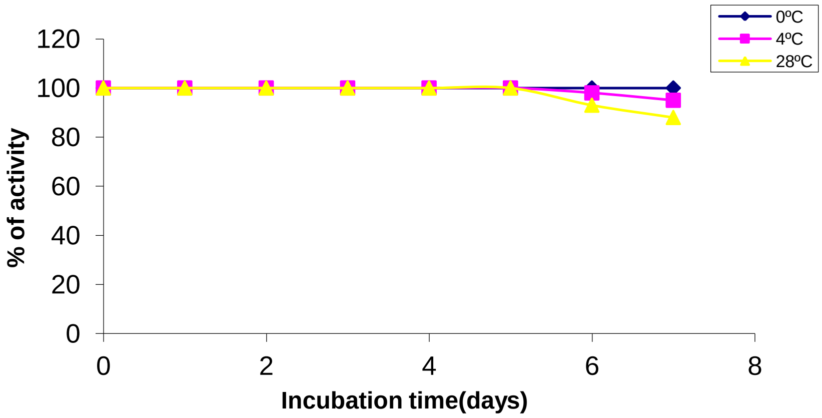

Fig. 6. Stability of the extracellular xylanase of *A. pullulans* at different temperatures

Table 3. Effect of the initial pH on the growth and production of extracellular polygalacturonase and xylanase[a]

	a. Polygalacturonase		
Initial pH	Growth (mg of dry weight)	Extracellular protein(µg/mL)	Polygalacturonase activity[b]
4.0	133.2	45.2	8.6
5.0	141.8	39.6	12.8
5.5	32.9	24.4	16.44
6.0	135.4	36.0	13.4
7.0	84.8	28.0	0.00
	b.- Xilanase		
Initial pH	Growth (mg of dry weight)	Extracellular protein(µg/mL)	Polygalacturonase activity[b]
4.0	394.4	48.6	28.7
5.0	544.2	48.0	22.9
5.5	382.0	46.0	94.6
6.0	532.5	37.2	15.23
7.0	360.0	68.8	47.48

a.- 7 and 9 days of incubation at 28°C, 100 rpm, with polygalacturonic acid or xylan as carbon source, and 5.28 g/L of glutamic acid for polygalacturonase and 5.28 g/L of ammonium chloride for xylanase as nitrogen sources.
b.- As milligrams of galacturonic acid/min/mg of protein.
c.- As milligrams of xylose/min/mg of protein

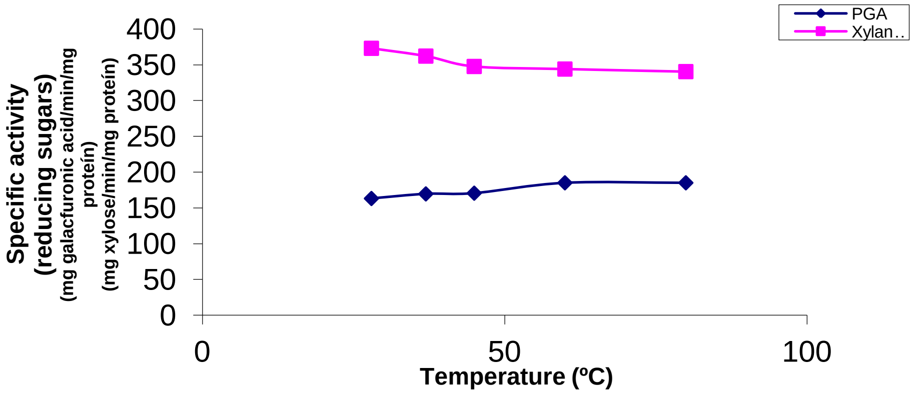

Fig. 7. Effect of incubation temperature on the enzymatic activities of polygalacturonase and xylanases of the fungus *A. pullulans*

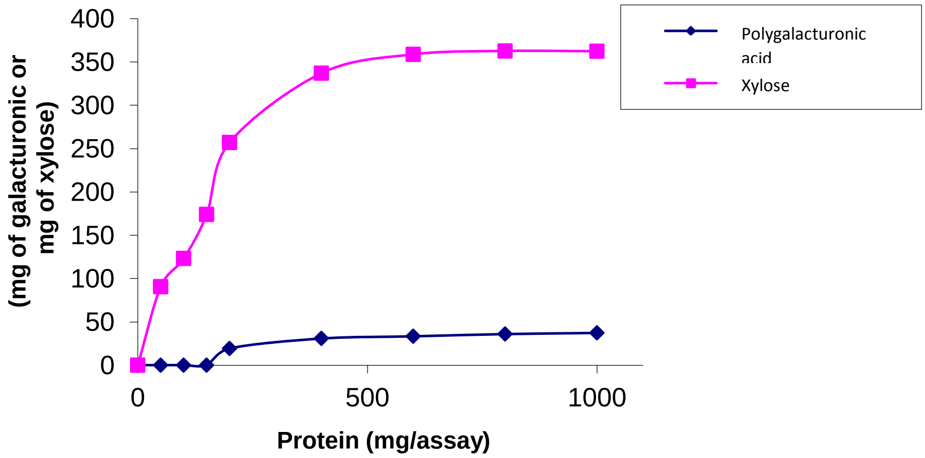

Fig. 8. Effect of the protein concentration on the extracellular enzymatic activities of polygalacturonase and xylanases

3.6 Effect of Incubation Time on the Enzymatic Activities of Polygalacturonase and Xylanases

Aliquots of 1.0 mL (100 µg/assay) of the extracellular lytic activities were incubated with the corresponding substrates, at 28°C. At different time intervals, aliquots of 100 µL were taken and the activities of polygalacturonase and xylanase were determined as described in material and methods. The results obtained are shown in Fig. 9. For the activity of polygalacturonase, the reaction was linear up to 60 minutes of incubation, while for xylanase it was up to 8 hours. In the literature was report 30 minutes for an endo-1, 4-β-xylanase production by *A. pullulans* using agro-industrial residues, 2.2 hours for a cellulase-free xylanase from *T. inhamatum* [59], 30 minutes for a *T. inhamatum* strain cultivated in liquid medium with oat spelts xylan [59], and 1 hour for the the fungus *P. citrinum* was successfully synthesized and expressed in the yeast *P. pastoris* retaining more than 80% of the original activity after 24h [60].

Finally, Table 4 shows a summary of the results of the characterization of the extracellular lytic activities of *A. pullulans*, observing that they have different induction times, different more efficient nitrogen sources, and are very stable at both 0°C and 4°C.

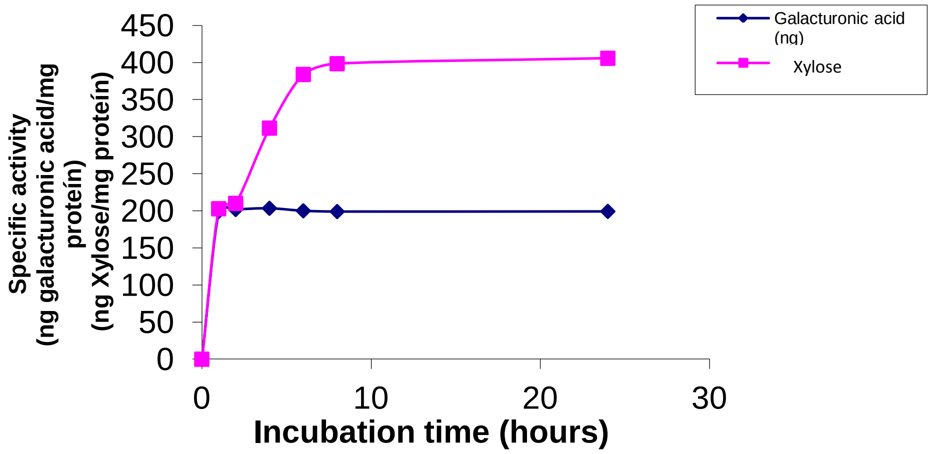

Fig. 9. Effect of the incubation time on the extracellular enzymatic activities of polygalacturonase and xylanases

Table 4. Summary of the induction characteristics of extracellular Polygalacturonase and Xylanase activities of *A. pullulans*

Parameter	**Polygalacturonase**	**Xilanase**
Growth (dry weight)	15 days	15 days
Maxim induction time	7 days	9 days
Nitrogen source	glutamic acid	Ammonium chloride
Initial pH	5.5	5.5
Stability at 0°C	73%, 7 days	100%, 7 days
Stability at 4°C	71%, 7 days	95%, 7 days
Stability at 28°C	43.1%, 7 days	88%, 7 days
Optimum temperature	60-80°C	28°C
Saturating enzyme concentration (µg/mL)	600	400
Incubation time	1 hour	8 hours

Other enzymatic activities related with this fungus have also been reported, such as: laccases [25], pullulan [26], cellulase [27], esterase [28], pectinases [29], with different applications, too is used as bioagent biocontrol [61], synthesis of compounds which possesses strong immunostimulatory activity [62], fermentative production of fructo-oligosaccharides [63], production of polymalic acid (PMA), a homopolymer of L-malic acid (MA), which has unique properties and many applications in food, biomedical, and environmental fields [64], and the some metal ions biosorption [65].

4. CONCLUSION

1.- The fungus shows polygalacturonase and xylanase activity.
2.- The polygalacturonase activity has a maximum induction time at 7 days, while that of xylanase has an optimal induction time at 9 days of incubation at 28°C.
3.- The xylanase activity was very stable at 4°C and 28°C, since it retains an activity of 100% at both temperatures after five days of incubation, while the polygalacturonase conserves 82.5% of its initial activity at 4°C, and 56.7% at 28°C.
4.- Glutamic acid was the nitrogen source that induced the highest polygalacturonase activity, and ammonium chloride for xylanase.
5.- The optimal induction pH was 5.5 for both activities.
6.- Maximum activity was detected at 60-80°C for polygalacturonase and 28°C for xylanase.
7.- Enzyme activity was directly proportional to enzyme concentration up to 600 µg/mL for polygalacturonase and 400 µg/mL for xylanase activity.

8.- Regarding the incubation time, the results obtained indicate that for the two activities analyzed, the reaction was linear until 60 minutes, for polygalacturonase and 8 hours of incubation for xylanase.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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