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Full Length Research Paper

Scrutiny for a disaccharide oxide reductase from filamentous extracellular pathogens

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World demand to find microorganism producers of enzymes with a potential biotechnological use is increasing daily. Therefore, the objective of this study was to find one or more microorganisms capable of oxidizing sugars. Of the thirteen strains tested AFG2 *Colletotrichum lindemuthianum* was the one who showed the best results concerning oxidant capacity against a variety of disaccharides. This fungus was selected for the time course of production of an extracellular oxide-reducing enzyme in liquid minimal medium. A disaccharide-oxidizing peak was obtained on the tenth day of incubation. It was found that the oxide-reducing activity is of enzymatic nature given that the extracellular extract is exposed to enzyme inhibitors and various denaturing agents. Additionally, it was possible to visualize the oxide-reducing activity by zymogram which allowed us to identify a band of enzyme activity with a calculated molecular mass of 79.43 kDa. Thus, *C. lindemuthianum* AFG2 wild fungal isolate is a candidate to study the production of these enzymes under optimized growth conditions that can increase performance.

Key words: *Colletotrichum lindemuthianum*, disaccharide oxide reductase activity relative zymogram.

INTRODUCTION

There is a worldwide and domestic industrial demand for enzymatic preparations of purified enzymes that exceeds 15 billion dollars. This has led to the establishment of a great industrial and scientific competitiveness to obtain the best fermenting microorganism with the best and most stable enzyme for design efficient industrial processes with a simple, efficient, and economical molecular conversion. Filamentous organisms producers of extracellular enzymes are key players in this scientific competition.

The fungal extracellular enzymes that oxidize or reduce carbohydrates by exchanging one or two electrons represent one special case of biotechnological interest. Few species of microorganisms that are able to produce these enzymes are known (Henriksson et al., 2000). Fungal enzymes are often related to the degradation of biopolymers such as cellulose (Eriksson et al., 1974; Li et al., 1996). The biological function of thisenzyme class for food uptake, phytopathogenesis or in the life cycle of fungi is not entirely clear. From the polysaccharide metabolism, glucosyl-glucose disaccharide such as maltose, isomaltose and cellobiose are producing. The cellobiose is a glucose dimer linked by a β(1,4)-glycosidic bond and generated during the degradation of cellulose. The disaccharide is enzymatically hydrolyzed to glucose or oxidized to cellobionolactone, an unstable compound that yields cellobionic acid spontaneously; although a lactonase has been proposed for this transformation (Westermark and Eriksson, 1974; Ayers et al., 1978; Ander, 1994). The cellobionolactone is an enzymatic inhibitor of cellulases and β-glycosidases from basidiomycete *Phanerochaete chrysosporium* (Lymar et al., 1995; Igarashi et al., 2003), and a strong inducer for cellulose gene expression (Ilmen et al., 1997).

The enzymes that oxidize carbohydrates to produce the corresponding lactones are important biotechnological agents of clinical diagnostics and industrial biocatalysis:

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the glucose oxidase (GOX) has been used widely in analytical chemistry and the food industry and the cellobiose dehydrogenase and cellulases in combination were used for the hydrogen peroxide production in bleaching processes (Pricelius et al., 2010).

By its great potential in the pharmaceutical industry, there have been several efforts to obtain pure cellobiose, in large quantities at low production costs (Kim and Day, 2010). In the protective effect against apoptosis induced by butyric acid, cellobiose is a better adjuvant than fructooligosaccharides (Calabresse et al., 1993). Cellobiose administered orally reduces both the neural fat in the liver and total cholesterol (Yamazaki and Ichiro, 2008).

Another reference is that enzymatic products such as cellobionic acid, which is used in the pharmaceutical industry as effective retarding agent in the release of active ingredients (Meyer and Isaksen, 1995; Ghahfarookhi et al., 2004). Also, cellobionic acid derivatives have been proposed as contrast agents in magnetic resonance imaging of the kidney and for topical use (Colet et al., 2000; Yu and Van Scott, 2004). In fungal enzyme production the cellobionic acid induces the synthesis of cellulases in *Sporotrichum thermophile*, as well (Canevascini et al., 1979).

Disaccharide oxide-reductases and their metabolites offer a great potential in the development of biotech-nological processes that require the transfer of electrons. Therefore the objective of this study was to search the oxide-reducing activity of extracellular filamentous organisms when disaccharides are substrates to select the best filamentous microorganism oxide reducers.

MATERIALS AND METHODS

Reagents

All chemicals used were purchased from Sigma Aldrich USA. Fungal/oomycetal culture media and all components of culture media were purchased from Bioxon. The isolated microorganisms used in this work were species of: *C. lindemuthianum* AFG1, AFG2, AFG3 75A and 75B (from common bean), *C. acutatum* (from avocado fruit), *Botrytis cinerea* (from strawberry), *Fusarium oxysporum* (from strawberry), *Candida albicans* (from human being), wild fungal isolate (straw) and the oomycetes *Phytophthora cactorum* (from strawberry plant). *capsici* (from chili fruit), *cinnamomi* (from avocado tree). All species were sheltered in the microbial collection of the Instituto de Investigaciones Quimico Biologicas de la Universidad Michoacana de San Nicolas de Hidalgo.

Reactivation of growth of the wild filamentous organisms was tested. Spores and chlamydospores of fungal and oomycetes were rescued from the storage medium (mineral oil or a mixture of silica gel and casein). Monosporic cultures of wild microorganisms spread in Petri dishes containing potato dextrose agar (PDA) plus 0.1% Triton X100 at a temperature of 10-20°C.

Extracellular oxide-reducing activity assay. a) Screening of fungi and oomycetes with a capacity of extracellular redox. Fungal/oomicetal propagules or gems, spores and chlamydospores were inoculated on solid medium (depending on the species of fungus or oomycete) supplemented with the redox dye 1 mM 2,6-dichlorophenolindophenol (DCPIP) which changes from blue to colorless when it is reduced, 0.005% chloramphenicol, 1% cellulose and 20 mM cellobiose. Cultures were incubated for five days at room temperature. The colorless areas in the agar around the inoculums were measured. The colorless zone diameter was measured for the selection of microbial isolates. Microbial colonies with the larger colorless halo diameter on a blue background were indicative of higher oxide reducing activity.

Production of extracellular microbial components (metabolites, proteins). 5×10^8 spores/ml of *C. lindemuthianum* were inoculated in minimal medium as reported by Damian et al. (2010), supplemented with 0.5% glucose and incubated at 25°C, samples of 10 ml of culture were taken every 24 h for 8 days. Mycelium was discarded and the liquid growth medium was collected, then concentrated by lyophilization and desalted on Sephadex G10. This preparation was used to determine the oxide-reducing activity.

The enzymatic assay was performed using the method described by Ghahfarokhi et al. (2004). It measures the enzymatic activity of the disaccharide dehydrogenase (DDH) by decreasing absorbance of the reaction mixture at 600 nm during the first three minutes. The composition of the reaction mixture was: 2.5 mM Cellobiose, 2 mM DCPIP, 4 mM NaF, all components were dissolved in 10 mM phosphate buffer at pH 6.3. The reaction was initiated immediately by the addition of 80 to 100 µg extracellular protein. The slope value was used to determine enzymatic activity. The molar extinction coefficient (ε) of DCPIP was used when read at 600 nm at 25°C and pressure of 1,012 atmospheres; $\varepsilon = 1.5712 \times 10^4\ M^{-1} \cdot cm^{-1}$. The enzymatic activity was calculated as =[(slope value)·(volume reaction)/ ε· wave longitude]÷ mg protein, and is expressed as specific activity = mol/min•mg protein.

Nature of the extracellular oxide reducing activity of *C. lindemuthianum*

Heat was the denaturing physically agent. The extracellular extract was placed in a boiling water bath for ten minutes. A solution of 10% SDS was the denaturing chemical agent. It was mixed with the extracellular extract in 1:1 ratio, and incubated for ten minutes. At the end of both denaturalizing process, the oxide reducing activity was determined. Two different inorganic salts containing silver and zinc ions with a concentration range of 0.05 to 5×10^3 µM were used. To determine if the enzyme is sensitive to inhibition by end product, lactobionic acid and D-glucuronolactone was used in a concentration range of 5×10^{-8} to 50 mM and 0.005 to 500 mM, respectively. Also, cellobiose (0.1, 1, 10, 100, 200, 300 and 400 mM) was tested to determine the sensitivity to substrate. Only one concentration is plotted.

DDH molecular mass

To determine the molecular mass of DDH, a native polyacrylamide gel at concentration of 8% acrylamide, was prepared, which can separate proteins whose molecular mass is in the range of 36 to 94 kDa. All solutions used to prepare the gel and the sample was made according to the protocol of Ausubel et al. (1983). The extracellular enzyme extract (250 mg protein) was mixed with 20 mM Tris buffer containing 1% glycerol and 0.2 mg bromophenol blue in a 1:1:1 ratio (v:v:v).

For the electrophoretic separation 80 mM γ-amino butyric acid (GABA)/acetic acid buffer at pH of 4.8 reported by McLellan, (1982) was used. Molecular mass markers were bovine serum albumin (BSA) and urease, prepared according to manufacturer instructions. Proteins were visualized by staining with silver salts. The electrophoretic running of albumin and urease allowed to obtain a standard curve with a correlation coefficient r = 0.9908 which

Table 1. Effect of disaccharides on the extracellular oxide reducing activity of filamentous microorganisms.

No.	Fungus/oomicete wild species	Disaccharide (diametermm)			
		Cellobiose	Lactose	Maltose	Sucrose
1	Fungalwildisolated	16±2	15±2	12±1.3	14±1.3
2	*Botrytiscinerea*	16±2.64	16±1.75	14±0.8	14±2
3	*Candidaalbicans*	0	0	0	0
4	*Colletotrichumacutatum*	10±1.52	12±1	11±0.6	10±1.5
5	*C.lindemuthianumAFG1*	13±1.3	14±0.67	16±2.5	14±2
6	*C.lindemuthianumAFG2*	27±1.71	28±2.3	24±2.5	28±3
7	*C.lindemuthianumAFG3*	16±2	16±2	16±0.9	16±1.5
8	*C.lindemuthianum75A*	18±1.5	18±1.7	18±0.6	15±1.5
9	*C.lindemuthianum75B*	16±1	18±1	15±2.3	13±1
10	*Fusariumoxysporum*	14±1	14±0.9	11±1.15	16±1.1
11	*Phytophthoracactorum*	0	0	0	8±1.5
12	*P.capsici*	0	0	0	0
13	*P.cinnamomi*	0	0	0	0

The results are averages of three independent experiments with three replicates each.

was used to calculate the molecular mass of the DDH.

The DDH activity was visualized by the method reported by Ludwig et al. (2004). Briefly, after concentrating the protein from three liters of culture medium, aliquots at 1.5 ml (250 mg protein) was used to visualize and determine the molecular mass of the DDH by electrophoresis. The gels were washed two times with water under constant agitation for 15 min. The gels were incubated with 10 mM acetate buffer at pH 5.5, containing the redox dye 3 mM DCPIP until that gel was uniformly stained in blue. DCPIP solution was discarded and then a few drops of a solution of 300 mM cellobiose were added on the surface of the gel for the development of colorless or pinkish-white bands corresponding to enzymatic activity.

Data analysis

Data were expressed as mean ± standard error (ee). It was tested by means comparison Tukey (α = 0.05), and an analysis of variance to determine the significant differences between treatments using the statistical software Stadistic version 7.0.

RESULTS AND DISCUSSION

Screening of mycelial microorganisms with extracellular oxide reducing activity

In the agar plate, the colorless areas around the fungal inoculate are consequence of reduced DCPIP during the oxidation of carbohydrate by microbial components excreted into the culture medium. The colorless areas around the fungal colonies with all the carbohydrate tested had a range that goes from 10 to 28 mm in diameter. Nine of the thirteen isolated filamentous microorganisms evaluated showed ability for oxide-reducing DCPIP in the presence of a disaccharide (Table 1). The microorganisms were subjected to grouping by correspondence analysis to select the best oxide-reducing activity producers in the presence of disaccharides. It was found that the DCPIP was reduced with all the disaccharides tested, equally, by *B. cinerea*, *C. lindemuthianum* AFG2, 75A and *F. oxysporum*. Isolates of *C. lindemuthianum* AFG2 and 75A had the highest activity with the disaccharide, cellobiose, lactose, maltose and sucrose (Figure 1).

The observation that filamentous fungi were able to reduce the DCPIP using the cellobiose was interpreted as that oxide-reducing activity that contributes to the production of metabolites necessary for the growth from polysaccharides such as hemicellulose and cellulose. Moreover, the yeast *C. albicans* and oomycetes were not able to reduce the DCPIP indicating that these organisms do not produce extracellular oxidoreductases that could oxide the cellobiose at the time of the last sampling. In the case of *C. albicans*, the absence of oxide reducing activity can be explained because in the medium in which it develops as a pathogen it does not require splitting the disaccharide cellobiose to its monomeric component, glucose, or it does not use the cellobionolactone nor cellobionic acid. In the case of oomycetes it is difficult to find an explanation for the absence of extracellular oxide reducing activity and for which further experimental work is required. This reason can be one of the differences that they have with filamentous fungi. It has not been ruled out that all microorganisms tested are using other substrates to reduce the DCPIP, so an experimental design is underway to explore this possibility.

Enzymatic nature of the extracellular oxide-reducing activity of *C. lindemuthianum* AFG2

Of the oxide reducing activity scrutiny, *C. lindemuthianum* AFG2 were selected to continue the study because it was

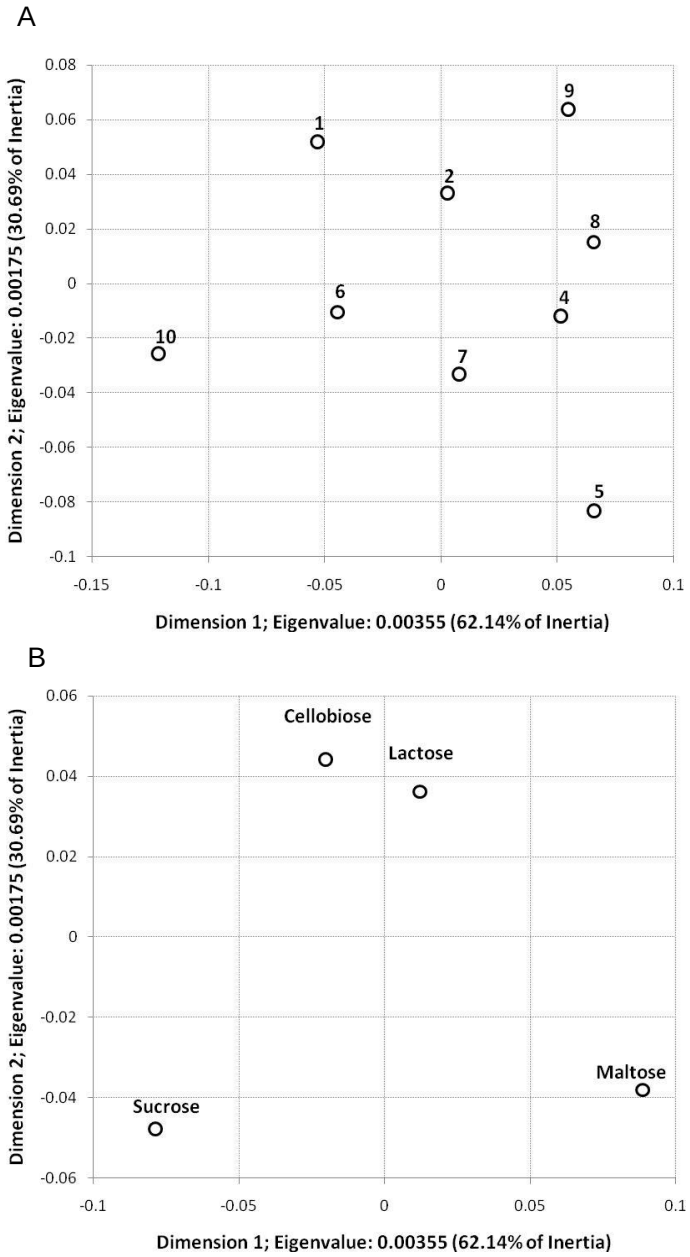

Figure 1. Correspondence map of the fungal oxide-reducing activity. (A) Association between the variables fungal growth, oxide reducing activity and fungus/oomicete. (B) Discrimination with disaccharides.

the one most active. There are two possibilities, one chemical and other enzymatic, that could cause DCPIP reduction in the presence of a disaccharide and therefore a dye change in medium that supports the fungal growth. To investigate the nature of the extracellular activity that causes DCPIP reduction, it was necessary to obtain a culture of *C. lindemuthianum* AFG2 in liquid minimal medium. The time course of production of the DDH was done and it was found that at ten days of incubation, enzyme activity reached a maximum of 16.45

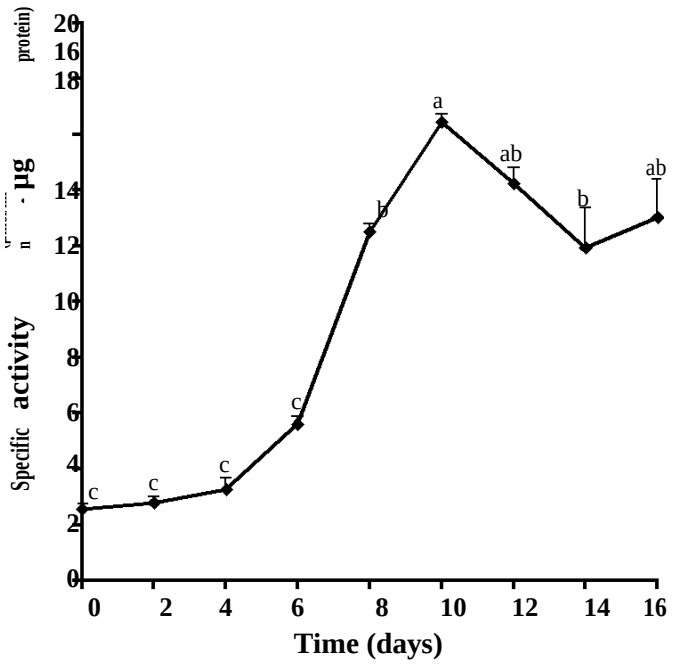

Figure 2. Timing course of DDH activity in *C. lindemuthianum* (AFG2) when it is grown with 0.5 % dextrose. The results are averages of three independent experiments with three replicates each. Tukey (α = 0.05).

µmoles/min·mg protein (Figure 2). The extracellular fungal protein extract was obtained from various liquid cultures, collected and concentrated to determine the enzymatic assays. This enzymatic source was exposed to denaturing treatments with 10% SDS and heat (boiling water) to measure the enzymatic activity.

Data of enzyme activity were compared with the activity obtained without denaturing agent. Figure 3 shows the results represented as relative activity, where 100% is the maximum value obtained under the experimental conditions. The maximum activity was obtained when the sample was not denatured, whereas in the presence of 10% SDS and heat, the activity obtained was 12.5 and 7.8% respectively, which represents 87.5 and 92.2% of denaturing. These results suggest that extracellular reducing activity DCPIP is due to an enzyme.

To verify the enzymatic nature of the oxide reducing activity, the extracellular extract was exposed to different compounds that inhibit it. Figure 3 shows the results of inhibition with 0.5 mM zinc sulfate, 0.5 mM silver nitrate, 2.5 mM lactobionic acid and 2 mM D-glucuronlactone. The relative enzymatic activity was obtained when the enzyme is not exposed to inhibitor, while that with the zinc and silver salts, we obtained an activity of 30.3 and 33.5%, representing a 69.7 and 66.5% of inhibition, respectively.

Lactobionic acid and of D-glucuronlactone inhibited the enzymatic oxide reducing activity until at 83.2 and 33.2%, respectively. This indicates that the enzyme is susceptible to inhibition by-products with lactobionic acid.

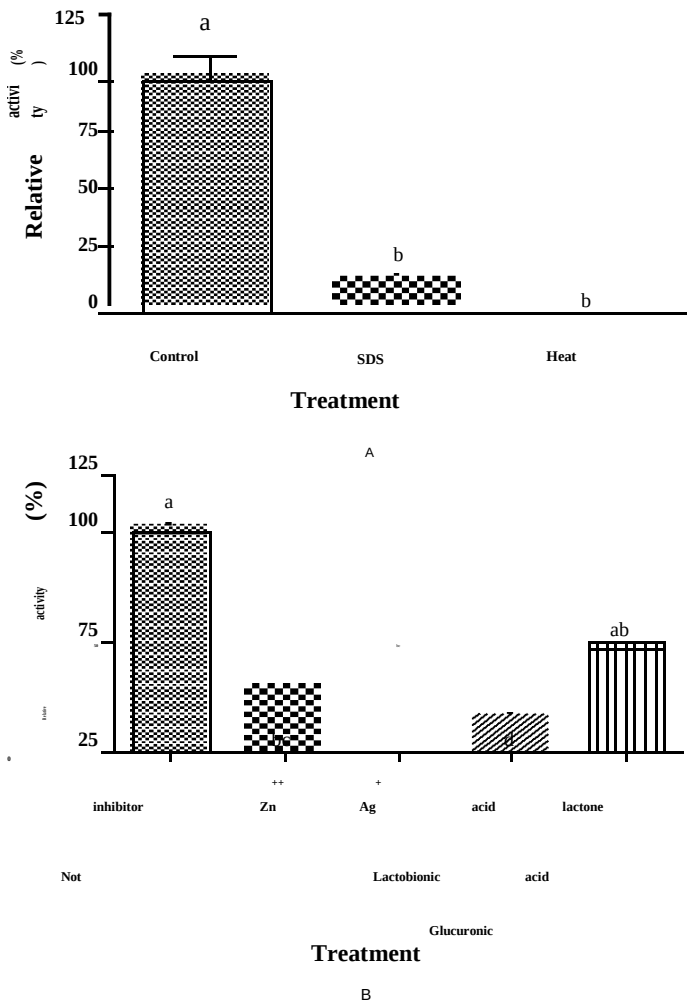

Figure 3. Enzymatic nature of the extracellular oxide-reducing activity from *C. lindemuthianum*. Extracellular protein treated with: (A) protein denaturing agents, and (B) metal inhibitors, substrate analogues or end product. The results are averages of three independent experiments with three replicates each. Tukey (α = 0.05).

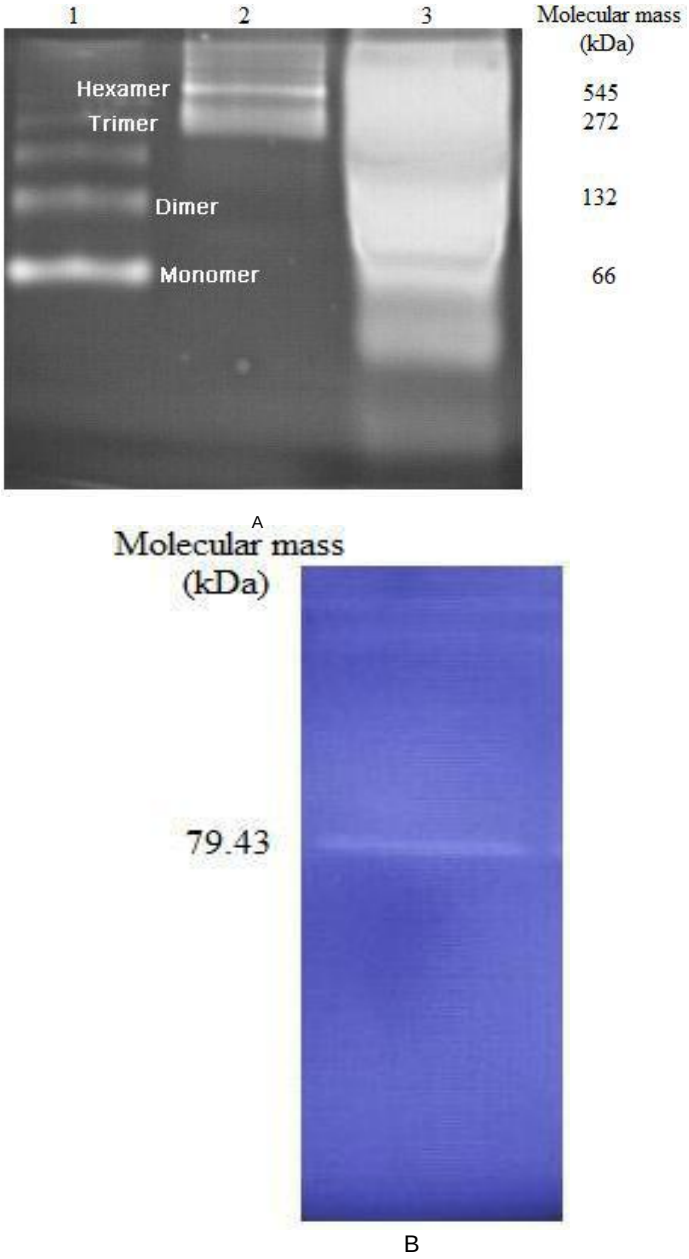

Figure 4. Molecular mass determination of disaccharide oxide reductase of *C. lindemuthianum* AFG2. Panel (A) shows an electrophoretic separation in native conditions of bovine serum albumin (lane 1), urease (lane 2) and the extracellular protein of *C. lindemuthianum*. Panel (B) is an image of the zymogram of this activity with the molecular mass detected and indicated by arrow. Between the panels the molecular mass of markers in kDa was indicated.

In *C. lindemuthianum* AFG2 isolates, it was determined that DCPIP reduction is effected by an extracellular enzyme that uses glucosyl-glucose disaccharides as substrates. Since the oxide-reducing activity was observed with all tested disaccharides, we named it as DDH, but cellobiose was used to determine enzyme activity in all following experiments.

Molecular mass of DDH from *C. lindemuthianum*

The results in Figure 4, shown on the panel B the gel image revealed DDH activity, there is clearly visible a colorless single band on a blue background with a calculated molecular mass of 79.43 kDa. The electrophoretic running of molecular markers is shown in the panel B. Lane 3 shows the running pattern of the extracellular protein from *C. lindemuthianum*. In this case we failed to detect DDH because there is no clear separation of the proteins, this was the result of an excessive amount of protein to be detected by silver staining, but also needed to be detected by enzyme activity. These results indicate that *C. lindemuthianum* produces a DDH enzyme. Comparing the results of this

study with those reported by Ghahfarokhi et al. (2004), it must be that *C. lindemuthianum* AFG2 produces oxide reducing enzymes with a value close to the average value reported in species of *Cladosporium*, a genus with species that over produce extracellular oxidoreductases. Based on the above and that *C. lindemuthianum* AFG2 has a partial characterization of cellulase (Flores Garcia personal communication), a further study of this pathogen was necessary to determine whether *C. lindemuthianum* is a candidate organism for production of DDH enzyme. Most investigations focused on this type of enzymes were carried out with basidiomycete *Phanerochaete chrysosporium* (Bao et al., 1993; Hernriksson et al., 2000). However, the yields of the oxide-reductases in the fermentation process are low (Meyer and Isaksen, 1995). Thus, the identification of organisms producing large quantities of a specific enzyme for simple processes and economics has been growing continuously due to various biotechnological applications that can be used. The detection of wild fungal isolates producing extracellular oxide reducing enzymes in simple growth medium and economics as used in this work allows evaluating a large number of organisms. It is concluded that *C. lindemuthianum* AFG2, a wild fungal isolate, is a candidate to study the production of these enzymes. Today we are determining its productive capacity in liquid culture media which provides the best fermentation conditions for higher performance in the extracellular oxidoreductases.

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