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Full Length Research Paper

Production and partial characterization of an exopolysaccharide from *Ustilago maydis* in submerged culture

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In Mexico, corn is widely cultivated and frequently parasitized by *Ustilago maydis*, a basidiomycete dimorphic fungus that causes the disease known as corn smut. It is widely consumed and is considered a culinary delight. From a biotechnological perspective, *U. maydis* offers the possibility as a producer of various metabolites, such as laccase and tyrosinase enzymes, glycolipids, and indole derivatives that can be used in pharmaceutical, cosmetic and food, due to its safety. The exopolysaccharides (EPS) are extracellular sugar compounds synthesized by a variety of microorganism and have shown various properties and applications. Among them are the improved rheology, texture, stability and mouthfeel of fermented dairy products. In addition, these compounds have shown biological activity as an antitumor agent, immune-stimulating and reducing blood cholesterol. The production of EPS by *U. maydis* was studied by culturing the cells in yeast extract-peptone-dextrose (YPD), YPD supplemented with $CuSO_4$ (YPD_T), Czapek Dox-sucrose, Czapek Dox-glucose, Czapeck Dox culture media with corn steep liquor and in another media containing high oil oleic sunflower as the sole carbon source. The presence of activity of glycosyltransferase by sodium dodecyl sulfate-polyacrylamide gel electrophresis (SDS-PAGE) was detected as well as the polymers formed by dinitrosaliscilic acid (DNS). The products of fermentation were characterized by enzymatic hydrolysis using carbohydrases, gel permation chromatography and atomic force microscopy.

Key words: *Ustilago maydis*, glucans, polymerization, glycosyltransferase, exopolysaccharide.

INTRODUCTION

Ustilago maydis is a basidiomycete dimorphic fungus which causes the disease known as corn smut in *Zea mays* and *Zea diploperenis*. This occurs when the fungus is in its dikaryotic fungus phase, producing infected bodies or ears. It is called *huitlacoche* in Mexico, where it is consumed as a culinary delicacy.

When cultured in the laboratory, *U. maydis* grows, forming haploid cells called sporidia (Banuett and Herskowitz, 1996; Juarez et al., 2011). This fungus, as

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well as *Candida bombicola, Schizonella melanogramma and Geotrichum candidum*, is a producer of two extracellular glycolipid biosurfactants: 1) manosileritritol lipids or ustilipids (MEL), 4-O-β-D-mannopyranosyl-D-erythritol which is esterified with short and medium size fatty acid chains (C2 to C8 and C10 to C18, respectively) as well as acetyl groups and 2) cellobiose lipids also known as ustilagic acid in which the disaccharide is attached through an o-glycosidic bond to a ω-hydroxy group of the 2,15,16-trihydroxy-o 15.16 – dihydroxi-hexadecanoic acid (Spoeckner et al., 1999; Hewald et al., 2005; Bolker, et al., 2008; Cortés et al., 2011).

Exopolysaccharides (EPS) are formed by various types of sugars. They may also contain proteins and are synthesized by the combined action of different types of glycosyltransferases (GTFs) as in the case of β-glycans (Pacheco et al., 2006), or are produced from a single substrate such as sucrose, thus producing glycans and fructans (Van Hijum et al., 2006). Since these compounds are excreted to the culture media, bioseparation operations aimed at their purification do not necessarily include cell breakage.

Biological glycans are constituted from a single carbohydrate unit to complex polysaccharides arrangements such as the chitin and β-1,3 glycans which are structural component of cell walls of fungi. In *U. maydis*, the chitin is the main structural component of cell walls and is synthesized by UDP-glucose β(1,3)-D-glucan and β(1,4)-D-glycosyltransferase (Klutts et al., 2006). Glycosyltransferases are enzymes (EC 2.4) that catalyze the transfer of a monosaccharide from an activated nucleotide sugar to specific acceptor molecules, forming glycosidic bonds. There are descriptions on the activity of glycosyltransferases (GTFs) (ß-glucan synthetase) associated to cell membranes that are able to produce glycans constituted by 60-80 residues of glucose by using UDP-glucose as substrate Douglas (2001). However, there are no reports on the activity of glycosyltransferase by *U. maydis* and on the consequent production of a polysaccharide.

Exopolysaccharides (EPS) are long-chain molecules produced and excreted mainly by bacteria and microalgae. EPS consist of branched units of sugars or sugar derivatives (Patel et al., 2011) which occasionally contain proteins and are synthesized by the combined action of different GTFs as in the case of β-glycans (Pacheco et al., 2006) or are produced by a single substrate by the action of sucrose, thus producing glycans and fructans (Van Hijum et al., 2006). EPS play a vital role in the protection of the microbes from adverse conditions such as water and osmotic stresses, nutrient shortage, presence of toxic compounds, bacteriophages, and to the action of antagonists (Looijesteijn et al., 2001; Patel et al., 2011; Donot et al., 2012). Microbial EPS can be classified into two groups based on their composition of monosaccharides and on the biosynthetic pathways involved in their production (Jolly et al., 2002). The homopolysaccharides (HoPS) include, dextran, mutan, alternan, reuteran, pullulan, levan, inulin, curdlan, etc., and consist of identical monosaccharides such as D-glucose or D-fructose and can be divided into two major groups: glucans and fructans. On the other hand, the heteropolysaccharides (HePS) comprise gellan, xanthan, and kefiran (Monsan et al., 2001; Patel et al., 2011; Donot et al., 2012). The biosynthesis of EPS includes three main steps: 1) assimilation of a carbon substrate, 2) intracellular synthesis of the polysaccharides and 3) EPS excretion of the cell (Vandamme et al., 2002). *Lactobacillus casei* CG11 cultured using various carbon sources such as glucose, sucrose, maltose, and lactose at a concentration of 20 g/L, showed a production of 160, 50, 60, 45 mg/L of EPS, respectively showing that the carbon source had an effect on EPS production (Cerning et al.,1994). Leifa et al. (2007) reported that the fungus *A. brasiliensisi* produced 2.3 mg/ 50 ml of EPS using glucose as carbon source and 3.4 mg/ 50 ml using sucrose. The same microorganism, when using yeast extract as a nitrogen source produced 9.4 mg/ 50 ml of EPS and 2.2 mg/ 50 ml when using peptone. The yield of EPS by lactic acid bacteria (LAB) depends on the strain and on the culture conditions such as temperature, concentration of oxygen, pH, and time of incubation. For achieving appropriate yields, a deep understanding of biosynthetic pathways are involved, and their relation to biological structures is mandatory (Patel et al., 2011).

One of the most important characteristics of EPS from basidiomycetes and other microorganism such as LAB is their action as antimutagenic, hypoglycemic, hypocholesterolemic, anti-inflammatory, antitumor, antiviral, bactericides, antiparasitic and immunomodulator agents (Selbman et al., 2002; Patel et al., 2011). In this category, it is possible to find various β-glycans such as scleroglucan (Selbman et al., 2002), botryospheran from *Botryosphaeria rhodina* (Miranda et al., 2007) and a glucan having anti-inflammatory action from *Collybia dryophila* and *Lentinus edodes* having β (1,3) and β(1,4) bonds (Pacheco et al., 2006). The objective of this work was to produce and carry out a partial characterization of an exopolysaccharide from *U. maydis* in submerged culture using different carbon sources.

MATERIALS AND METHODS

Biological material

U. maydis FBD12 (diploid strain) used for this work was kindly donated by Ms. Flora Banuett from California State University, USA, and kept in 50% [v/v] glycerol solution at -70°C. Strain was cultured at 32°C in yeast extract-peptone-dextrose broth (YPD), using Erlenmeyer flasks and in conditions of agitation, using an orbital shaker at 200 rpm until culture reached stationary phase (48 h). Initial concentration of the inoculums was 3 g/L. Culture media YPD and YPD supplemented with $CuSO_4$ (YPD_T) as well as Czapek Dox

added with glucose, sucrose or corn steep liquor culture media were used for the detection of glycosyltransferase. Culture media containing oil was used for the detection of the polysaccharide (Spoeckner et al., 1999).

Culture media composition

For yeast extract-peptone-dextrose broth (YPD), yeast extract (10 g), peptone (20 g), and dextrose (20 g) were dissolved in distilled water (1 L) and 15 g of bacteriological agar were added (Ausubel et al., 1994).

For Czapek Dox medium, dextrose or sucrose (30 g) or corn steep liquor (CSL) (0.6 ml), $NaNO_3$ (3 g), K_2HPO_4 (1 g), Mg SO_4 (0.5 g), KCl (0.5 g), $FeSO_4$ (0.01 g) were dissolved in 1 L of distilled water.

Medium with addition of oil for the production of glycolipids (SWNL) was prepared by using the following compounds (Spoeckner et al., 1999): K_2HPO_4 (1 g), citric acid (0.2 g), $MgSO_4$ (0.4 g), $FeSO_4$ (0.03 g), corn steep liquor (CSL)(0.6 ml), $CaCO_3$ (1.5 g), NH_4SO_4 (1.3 g), and sunflower oil with 75% oleic acid (20 ml) which were dissolved and dispersed in 1 L of distilled water. CSL was added to YPD and Czapek Dox culture media instead of dextrose or sucrose, to to find out possible inducing effects of this ingredient on the production of the EPS.

Preparation of an extract containing glycosyltransferase and polysaccharide

Cells were separated by centrifugation from media SWNL at 3, 5, 7 and 9 days of fermentation. Supernatant was subjected to fractioned precipitation with $(NH_4)_2SO_4$ until reaching 90% saturation and centrifuged at 10000 $\times g$ for 30 min and cells were re-suspended in phosphate buffer (1 mM, pH 6.8) containing phenylmethylsulphonyl fluoride (PMSF). Then, the suspension was concentrated by using an Amicon ultrafilter at 4°C, using a 10 kDa cut off membrane in a nitrogen atmosphere. Obtained extract (EC) or retentate was used to measure the activity of glycosyltranferase with 3, 5 dinitrosalicylic acid (DNS) (Miller, 1959). All experiments were made in triplicates.

Determination of protein

Protein was evaluated following the Bradford (1976) method, using bovine serum albumin (Sigma-Aldrich) to construct the standard curve and commercial Bradford reagent (Sigma-Aldrich). All experiments were made in triplicates.

Activity of glycosyltransferase

Glycosyltransferase activity was determined using dinitrosalicylic acid (DNS). Each sample was transferred to a 10 ml test tube; 1.5 ml of DNS were added, mixed with 50 µl of enzymatic extract and stirred by means of a vortex. Test tubes were covered with a cap and heated in boiling water for 15 min and immediately transferred to a cold water bath. 2 ml of deionized water were added to each tube and mix stirred in a vortex. Absorbance at 550 nm was registered using as blank, the buffer solution that contained the sample. All experiments were made in triplicates.

Molecular weight determination (PAGE)

Electrophoresis was carried out in polyacrylamide gels (7.5%). Applied power was 120 volts for 90 min and gels were stained with Coomassie blue G-250 and distained by using a mixture of methanol: acetic acid: water (3:1:6 v:v:v) (Bio-Rad Laboratories, Miniprotean II Instruction Manual). All experiments were made in triplicates.

Detection of glycosyltransferase by PAGE using Schiff staining reaction

Extract containing glycosyltransferase was separated using PAGE as previously described. Gel was incubated for 18 h at 37°C in a 0.2 M PBS buffer at pH 6 containing 1% sucrose and washed with distilled water and immersed in a 1% periodic acid for 10 min. After this, gel was washed with distilled water and stained with Schiff reagent for 25 min and washed out three times with acetic acid solution (7%) and kept for 24 h in the absence of light. Positive reaction indicating the existence of glycosyltransferase was associated to the presence of pink bands (Jung et al., 2007). All experiments were made in triplicates.

Determination of molecular weight of the polysaccharide

To evaluate the possible formation of polymers by the catalytic action of glycosyltransferase, the extract was incubated with dextran blue 2000, UDP-glucose and dextran blue 2000 + UDP-glucose as initiators of polymerization with and without (blank) 1% sucrose solution in 0.2 M PBS pH 6 buffer for 18 h. The final extract (500 µl) was added with 120 µl of the extract, 370 µl of sucrose solution, 10 µl of dextran blue and 10 µl of UDP-glucose; the final volume was adjusted to 1.5 ml with buffer solution. For the blank samples, buffer solution was used instead of sucrose solution.
When reaction was completed, proteins were precipitated with 40% trichloroacetic acid (TCA) and resulting solution was filtered and analyzed in a Zetasizer nano (Malvern Instruments Zetasizer Nano Apllication Note MRK577-01) at 25°C to evaluate possible presence and size of extracellular materials (Dextran blue 2000 and UDP-glucose were used as standards). All experiments were made in triplicates.

Characterization of the polysaccharide by gel permeation chromatography (GPC)

Molecular weight determination and partial characterization of the polysaccharide was carried out in an HP Polymer Laboratories PL-ELS 100 chromatograph, equipped with two fluid bed columns (GPC/SEC PL gel 10 µm MIXED-BLS 300 × 7.5 mm) arranged in serial mode and Cirrus software was used for data processing. The nebulization and evaporation chambers where operated at 40 and 80°C respectively. Calibration was made with polystyrene standards and the molecular weights ranged from 162 to 6,000,000 kDa. Mobile phase was tetrahydrofurane (Merck THF), flow to flow rate was 1.0 ml/min and volume of injection was 20 µl. All experiments were made in triplicates.

Enzymatic hydrolysis

Characterization of polysaccharides was carried out by enzymatic hydrolysis using ß-glucanase from *Trichoderma longibrachiatum* (CELLUZYME from ENMEX); ß-glucosidase (G-4511 Sigma); mixture of β-glucanase and β-glucosidase; lipase (L-1754 Sigma) and bacterial alkaline protease from *Bacillus licheniformis* (DETERZYME® L-660 from ENMEX). The final volume of each test

Table 1. Enzyme activity in the raw extracts from the fermentation in all the culture media.

Culture medium	Glycosyltransferase activity (µmol of glucose $ml^{-1}min^{-1}$)	Protein (mg ml^{-1})	EAU mg^{-1} of protein
YPD	0.844±0.042	0.472±0.023	1.79±0.08
YPD_T	0.0436±0.002	1.216±0.06	0.03±0.001
YPD CSL	0.94±0.047	2.2±0.110	0.422±0.02
Czapek Dox with glucose	0.0704±0.02	0.534±0.026	0.132±0.006
Czapek Dox with sucrose	1.32±0.066	1.33±0.066	0.99±0.04
CZ CSL	1.11±0.055	2.1±0.10	0.52±0.02
SWNL 3º	0.29±0.014	4.2±0.21	0.07±0.003
SWNL 5º	0.99±0.049	4.8±0.24	0.208±0.01
SWNL 7º	2.02±0.10	7.0±0.35	0.29±0.014
SWNL 9º	0.66±0.033	2.2±0.11	0.297±0.014

YPD= Yeast extract, peptone and dextrose broth; YPD_T= YPD with copper sulfate; YPD CSL= YPD with corn steep liquor; CZ CSL = Czapek Dox with corn steep liquor; SWNL = broth used for the production of glycolipids analyzed after 3, 5, 7 and 9 days of fermentation and in triplicate each. Results are the average of three determinations ± standard error.

was 200 µl and contained 198 µl of extract and any of the following enzymes: 2 mg of ß-glucanase, 6 mg of ß-glucosidase, 2 mg of lipase and 1 mg of protease (Pacheco et al., 2006). Reactions with β-glucanase and β-glucosidase were carried out at 50°C for 1 h in 0.1 M, pH 5 citrate buffer while reactions with lipase were carried out at 37°C for 60 min in 0.1 M, pH 8 phosphate buffer. Reactions with protease were performed at 50°C for 2 h in PBS 0.1 M, pH 8 buffer. Blank samples did not contain enzymatic extract. From each experiment, 120 µl of sample were added with 1 ml of HPLC purity tetrahydrofurane prior to analysis by gel permeation chromatography (GPC). All experiments were made in triplicates.

Atomic force microscopy

Polymers obtained from the extract and in various solvents were observed using Atomic Force Microscopy, Nanoscope IIIa (AFM), Digital Instruments Veeco, Santa Barbara California USA. For scanning samples, silicon nitride probes were used with a force of contact of 0.3 N/m. Two microliter (2 µl) of sample were used for the observation onto plates of muscovite ($KAl_2(Si_3Al)O_{10}(OH, F)_2$). Different observation areas were considered according to sample and magnification applied. Applied frequencies were 2 to 10 Hz per line and scanning was performed once polymer was detected. Images were captured from monitor in tagged image file (TIF) format for further analysis. Blank samples had the polymer dissolved in tetrahydrofurane (THF) which was used in GPC runs. Samples labeled "THF-water" were prepared by evaporation of THF and re-suspended in water. Samples labeled as "mixture" were treated with β-glucosidase and β-glucanase. Those samples labeled as "in acetone" are those that were treated with this solvent before GPC experiments to eliminate possible presence of proteins. Those labeled as "in chloroform" were treated with this solvent prior to GPC experiments to eliminate possible presence of proteins**.** All experiments were made in triplicates.

RESULTS AND DISCUSSION

Activity of glycosyltransferase in different culture media

The highest biomass production was observed in SWNL medium (18.1 g/L), followed by YPD_T (15.67 g/L), YPD (9.30 g/L), Czapek Dox-sucrose (4.1 g/L) and finally, Czapek Dox-glucose (3.7 g/L); this could probably be explained by a high lipase activity. Maximum glycosyltransferase activity was observed in YPD media (1.8 times activity obtained when using Czapek Dox-sucrose media), followed by that found in Czapeck Dox with corn steep liquor (CZ CSL) and, in descending order: YPD with corn steep liquor (YPD CSL) > SWNL at 5, 7 and 9 days of fermentation, and finally in Czapeck Dox media with added glucose (13.5 times less activity than YPD) (Table 1).

In the case of YPD, addition of Cu^{++} probably decreased the total protein production or inhibited enzyme activity. When using Czapeck Dox-sucrose, 3 times more enzyme activity was found than when Czapeck Dox-glucose media was used. This was possibly due to glucose acting as repressor of the synthesis of glycosyltransferase. Glucose has been reported to inhibit a number of microbial enzymes (Martins et al., 2006).

Enzymes detected from all culture media by zimograms in PAGE (with the exception of SWNL media) showed molecular weights above 212 kDa while the protein detected in SWNL medium had a molecular weight of 53 kDa. Possible inducing effects of CSL in Czapek and YPD media were studied given the presence of a 53 kDa band when using SWNL culture media. No effect of CSL was found and the presence of detected enzyme can be due to the presence of high oleic acid-sunflower oil in the media.In Table 1, it is possible to observe that the extract from CZ CSL had, after extracts obtained from YPD medium (1.79 EAU mg^{-1} of protein), the highest value of enzymatic activity (0.525 EAU mg^{-1} of protein), followed by the extract from YPD CSL (0.422 EAU mg^{-1} of protein) and the SWNL medium at 9 days of culture, which had 0.297 EAU mg^{-1} protein. The glycosyltransferase was detected in the culture medium SWLN at 3, 5, and 7 days

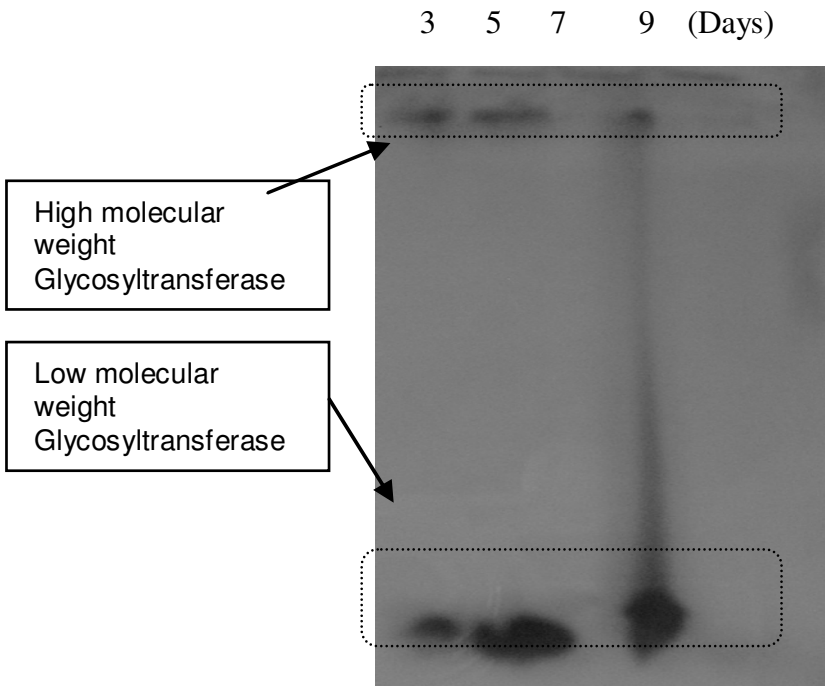

Figure 1. Detection of glycosyltransferase from *Ustilago maydis* in 7.5% PAGE stained with Schiff reagent. SWNL = broth used for the production of glycolipids analyzed after 3, 5, 7 and 9 days of fermentation.

of incubation. This was achieved by means of the reaction of the glycosil moiety of the enzyme with Schiff reagent in the electrophoresis gel. Results are shown in Figure 1. The absence of glycosyltransferase on the 9th day of culture is noteworthy. Reducing sugars measured at 3, 5, 7 and 9 days of fermentation were 0.07, 0.2, 0.29 and 0.30 EAU (enzymatic activity units) respectively. Those detected at 9 days of culture (Table 1) could be the product of the activity of other enzymes such as invertase, since glycosyltransferase was not present on that fermentation day.

The glycosidic bond of the glycoprotein (glycosyl-transferase) was split using β-glucanase and β–glucosidase. Products of the reaction were subjected to PAGE and no bands were detected when using Schiff reagent, which indicated that the product was originated from a glycoprotein.

Determination of particle sizes of glycosyltransferase reactions products

In Table 2, results of polymer formation under the different reaction conditions after protein precipitation with TCA (15%) are shown. It is noticeable that important changes on the hydrodynamic radius were observed in B and D samples, indicating that reaction proceeds with sucrose or sucrose and UDP-glucose. Regarding reaction with blue dextran, it was possible to observe the generation of low molecular weight compounds which may indicate the presence of fructose and glucose by the action of invertase or glycosyltransferase. All experiments were made in triplicates.

Two different species were detected according to evaluated hydrodynamic radius when they differ by a factor of 2, which corresponds to a factor of 8 in molecular mass. β-Glucans are present in cell walls; their physiological role is still unknown and approximately 80% of glucans are of the β-1,3 type. These compounds are synthesized within the plasmatic membrane and for glucan production, UDP-glucose is required as substrate and GTP as activator (Sakai et al., 2007). There are, however, studies in which exopolysaccharides are produced by fermentation processes such as the penta-saccharide produced by *Streptococcus thermophilus* formed with D-galactose and D-glucose units (Nordmark et al., 2005). There are also reports on the production of water-soluble extracellular structures composed of α-D-glucans (α-1, 3 glucan) by a glycosyltransferase of *Streptococcus sobrinus* using sucrose as a substrate (Cheetham et al., 1990).

Characterization of the polysaccharide by gel permation chromatography

In Figure 2a, peaks corresponding to polymers of different molecular weight obtained by fermentation in

Table 2. Hydrodynamic diameters of the glycosyltransferase reaction products under different reaction conditions.

Sample	Enzyme extract (µl)	1% sucrose buffer (µl)	Buffer without sucrose (µl)	UDP-Gluc (10 mg/ml) (µl)	Dextran (1 mg/ml) (µl)	Peaks of the polymer	
						Hydrodinamic diameter (nm)	Hydrodinamic diameter (nm)
A	120	370	-	-	10	178.9 (100%)	
B	120	370	-	10	-	330.6 (100%)	
C	120	360	-	10	10	224.7 (100%)	
D	120	380	-	-	-	169.2 (98.6%)	4996 (1.4%)
TA	120	-	370	-	10	255.3 (100%)	
TB	120	-	370	10	-	208.3 (100%)	
TC	120	-	360	10	10	202.7 (100%)	
TD	120	-	380	-	-	187.2 (100%)	

TA, TB, TC, TD: Blanks of samples A, B, C and D.

SWNL medium are shown. Four groups of polymers were classified whose molecular weights were within the range of 26862 to 65349 kDa with an average MW of 44285 for peak 1, and their average MW corresponded to the average MW´s of 983 and 439 Da for peaks 2 and 3 respectively. To carry out the characterization of the produced polysaccharides (peak 1), a series of hydrolytic procedures were applied as well as treatments with two different solvents to precipitate proteins and to be able to detect glucans. All experiments were made in triplicates.

Hydrolysis was performed by using β-glucanase, β-glucosidase, a mixture of β-glucanase and β-glucosidase, lipase, and protease, and, in addition, samples were treated with chloroform and acetone. The main peak observed in Figure 2a was broken down in a series of smaller peaks. Treatment with β-glucanase provided an important extent of hydrolysis along with a 14 fold reduction of initial concentration after treatment with β-glucanase. The hydrolysis by β-glucanase of *T. longibrachiatum* of these compounds suggested the presence of β- 1-3 bonds in the structure of the glucan, since this enzyme specifically hydrolyzes these bonds. Hydrolisis with β-glucosidase produced a smaller number of products than after treatment with glucanase. The extent of hydrolysis was half to that attained by β-glucanase.

The smaller extent of hydrolysis observed with β-glucosidase than with glucanase suggested the presence of a smaller number of homo β-1, 4 bonds and higher number of β-1, 3 bonds in the polysaccharide (Figure 2a). However, hydrolysis with a mixture of β-glucanase and β-glucosidase produced a synergic effect on the reaction and a different profile was obtained than when the single enzymes were used individually as shown in Figure 2b where 6 peaks were observed, being peaks 2, 3 and 4 of a higher intensity while peak 1 presented a better definition than when hydrolysis was carried out with a single enzyme, thus narrowing the interval of corresponding molecular weights and suggesting the breakage of the polymer by the two enzymes and the presence of a predominant polymer with a molecular weight of 40.225 kDa while the other peaks corresponded to lower molecular weight compounds.

Hydrolysis with lipase was carried out to break complex high molecular weight lipidic compounds formed with proteins, the polysaccharide and glycolipids. It is possible that the peak having a retention time of 16.8 min corresponded to a glycolipid since it disappeared from the chromatographic profile after the enzyme treatment. When performing the hydrolysis with protease, there was no drastic decrement of the intensity of the peaks with respect to the crude extract. To eliminate the protein from the glucan, chloroform and acetone were used. In the solvent treated samples, different products of the reaction including glucans (high molecular weights) and glycolipids were detected and probably, at a very low concentration. Also, when the sample was subjected to proteolysis, a minimum change in the profile of glycopeptides was noticed. These results are similar to the observations of other authors (Spoeckner et al., 1999).When carrying out submerged culture of *U. maydis*, it was found

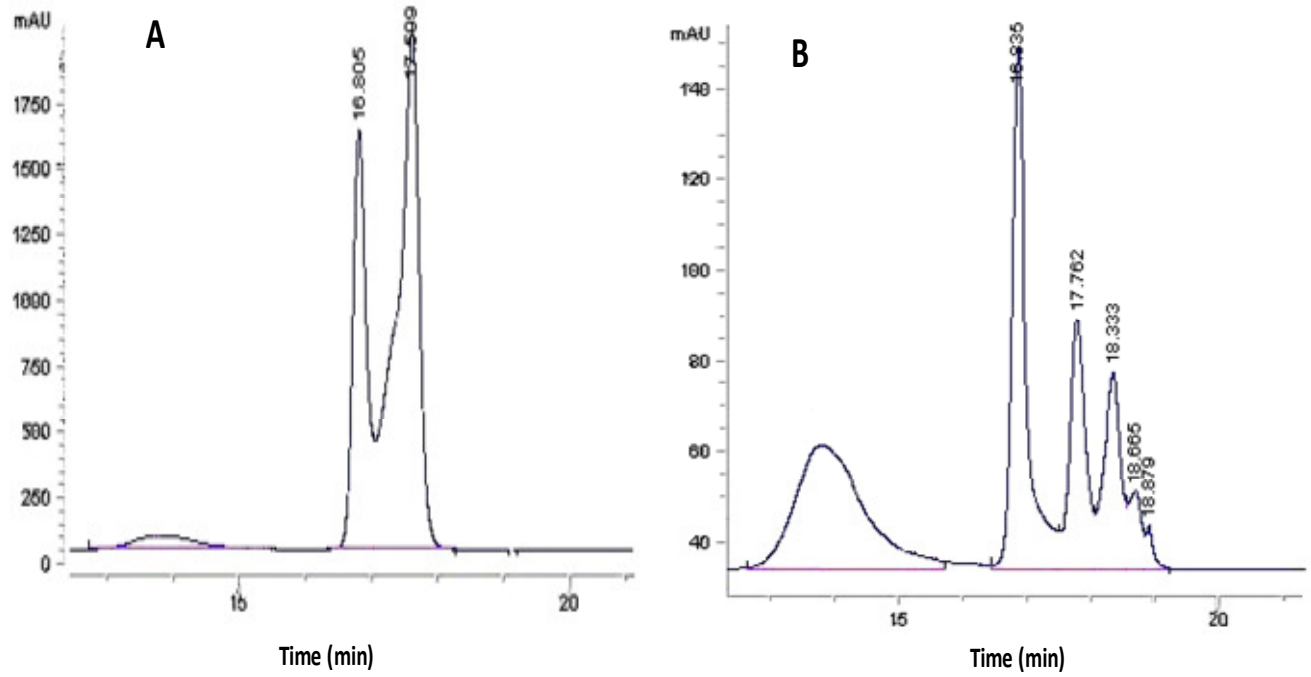

Figure 2. SEC chromatogram of polysaccharide extract produced by *Ustilago maydis* before (a) and after (b) enzymatic hydrolysis with carbohydrases.

that formation of polymers occurred during the stationary phase of the culture. This was opposed to findings when experimenting with other suspended cultures with other microorganisms (Webster et al., 2008). Type of products as well as expressed enzymes by *U. maydis* is highly dependent on the media used. In YPD medium, a high molecular weight enzyme was expressed and a polysaccharide was formed which increased the viscosity of the media during the concentration by ultrafiltration.

Characterization of polysaccharide by atomic force microscopy

Micro and nano characterization of the polymer was carried out by atomic force microscopy (AFM). In Figure 3a, it is possible to observe that original sample had patterns which are typical of this kind of polymeric structures with corpuscular and interlinked linear arrangements similar to those reported (Morgan et al., 1999) when observing a commercial glucan (Glucagel^{TM}) by AFM. Coexistence of corpuscular and linear morphologies has been reported by these authors in samples of the commercial glucan subjected to dehydration-rehydration processes such as those carried out for preparation of the sample for observation in the AFM. Aggregation due to thermal denaturation of glucans (Application Note Agilent Technologies, Inc.) has been reported, and also, those changes of pH and heating processes produced aggregation in glucans observed by AFM (Sletmoen et al., 2005).

Samples treated with a mix of β-glucosidase and β-glucanase (Figure 3b) when subjected to observation by AFM showed degradation, structural damage and formation of aggregates with sizes around 73 to 74 nm, which formed irregular and incomplete rings on the stage of the AFM. These findings support the idea of the existence of a main glucan having β-1,4 bonds and residues of hydrolytic action which would be formed mainly by glucans having β-1,3 bonds.

It was possible to identify carbohydrate polymers of different molecular weights in the extract obtained from the fermentation of *U. maydis* in SWNL culture media. Glucans having β-1,4 and β-1,3 bonds were detected, having molecular weights within a range of 26,862 to 65,349 Da, as well as others having molecular weight of around 44,285 Da. By means of AFM, the presence of structures with corpuscular and interlinked arrangements were found, which, after enzymatic treatment, showed degradation, structural damage and formation of small aggregates.

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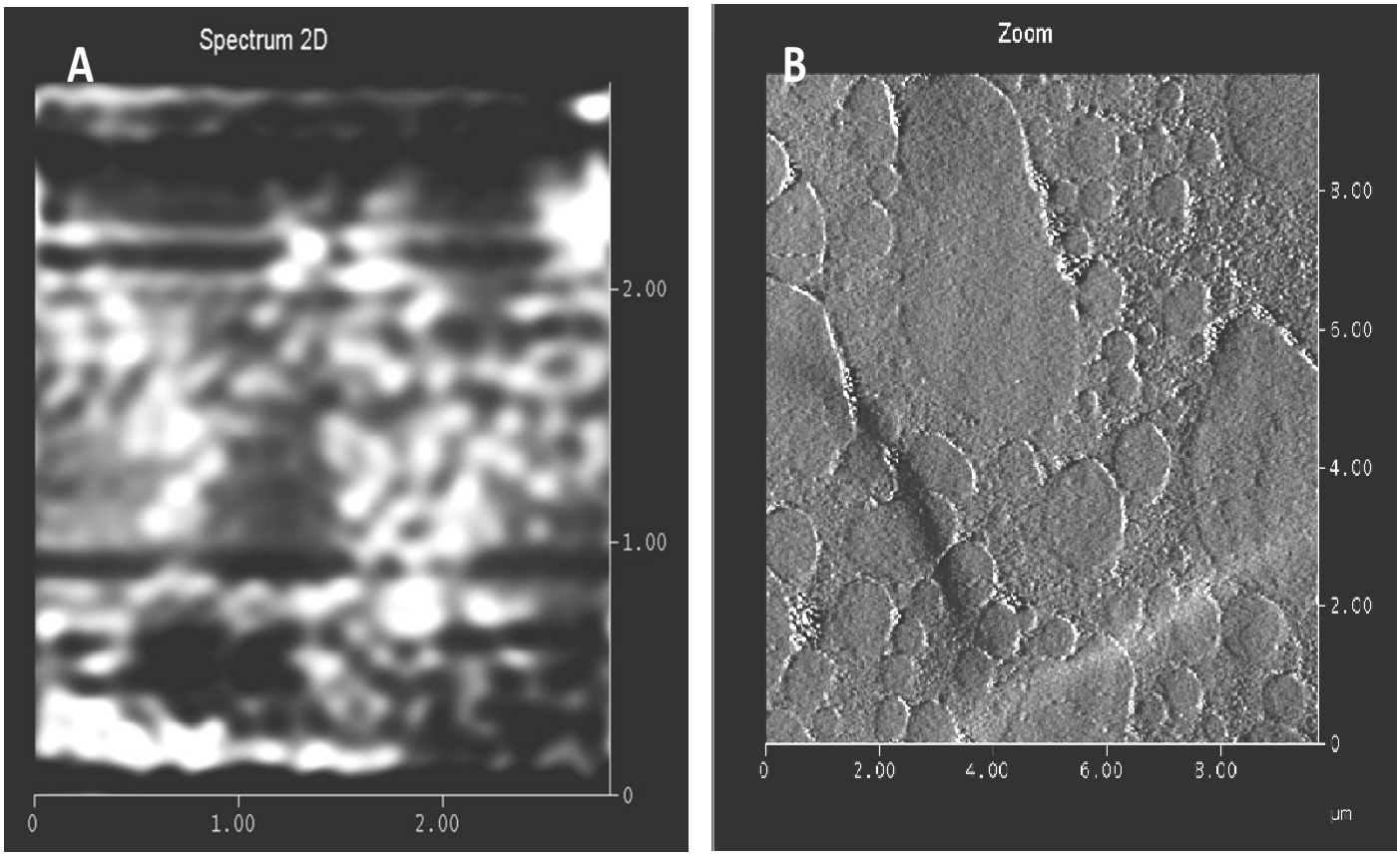

Figure 3. (a) AFM image of the β-glucan showing patterns typical of this kind of polymeric structures with corpuscular and interlinked linear arrangements; (b) AFM images of β-glucan treated with the enzymatic mix β-glucosidase and β-glucanase. Structures were disrupted and aggregates decreased size and changed pattern in respect to those shown in (a).

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