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Isolation and Identification of Novel Disaccharide of ∝-L-Rhamnose from *Penicillium chrysogenum*

Tarek A.A. Moussa and Dalia M.I. Ali

Department of Botany, Faculty of Science, University of Cairo, Giza 12613, Egypt

Abstract: The dry biomass and protein content in culture filtrate of *Penicillium chrysogenum*, increase with increasing the time of incubation. On the contrary, carbohydrate content of the culture filtrate was decreased with increasing the incubation time. The amount of exodisaccharide (EDS) after 5 and 10 days of cultivation periods was 0.6 and 2.5 g/l, respectively. The yield (g of biomass/g of reducing sugars) was obtained after 5 days (0.7 g/g) and 10 days (1.44 g/g), also the maximum EDS/biomass (g/g) ratio after 10 days was 0.17 g/g which is 2.13 times higher than in case after 5 days (0.08 g/g). The ^{1}HNMR spectrum is well representation of protons from gycosidic groups of carbohydrates. The signal at 5.261 and 1.244 ppm were assigned to the anomeric and methyl protons at position-6, respectively, of ∝-L-rhamnopyranosyl residues; also the signals at 3.895, 3.759 and 3.508 ppm were assigned to H_2, H_5 and H_4, respectively, of a-L-rhamnopyranosyl residues. FT-IR spectrum is consistent with those of typical carbohydrates. The *m/z* is at 289 with base peak 161 with relative abundance 100%. The EDS is thermally stable at all temperatures tested the temperature was increased by 100°C.

Key words: *Penicillium chrysogenum* . exodisaccharide . ^{1}HNMR . FT-IR . MS . TGA

INTRODUCTION

Disaccharides are formed when two simple sugar molecules bind together. Sometimes two similar kinds of simple sugars combine. Often, two different kinds of sugar molecules combine to form a disaccharide. Disaccharides are produced commercially by the incomplete hydrolysis of larger polysaccharides. An alternate process combines two monosaccharide sugars by means of a condensation reaction to form disaccharide sugars. Usually, disaccharide sugars must be hydrolyzed and split into their simple sugar components before they can be fermented.

Microorganisms, including wine yeasts, produce enzymes that can hydrolyze sucrose and when sucrose hydrolyzes, each sucrose molecule splits into one glucose and one fructose molecule. This process produces a 50-50 mixture of glucose and fructose monosaccharides called "invert sugar." Sucrose is a non reducing sugar and it cannot be accurately measured with Clinitest tablets.

Production of the artificial sweetener, lactosucrose, by various microorganisms containing levansucrase activity was investigated. Of the tested bacteria, *Bacillus subtilis* was the most effective producer using lactose as an acceptor and sucrose as a fructosyl donor. Lactosucrose production by this strain was optimal at pH 6.0 and 55°C [1]. It has been reported that disaccharides of heparin or heparin sulfate suppress tumor necrosis factor production in macrophages and IL-8 and IL-1 secretion by intestinal epithelial cells [2, 3]. These events suggest that GAG oligosaccharides have effects on cytokine expression. Moreover, it has been shown that high molecular weight hyaluronan (HA) inhibits NF-$_{\kappa}$ B activation [4], whereas low molecular weight HA induces NF-$_{\kappa}$ B activation [5]. This suggests that the cell biological activities of GAGs are dictated in part by their molecular size.

The microbial exopolysaccharides (EPS) are a class of high value biopolymers with a wide variety of industrial applications [6, 7]. In particular, many kinds of EPS have been produced from submerged cultures of mushrooms or enthomopathogenic fungi [1, 8-13]. Recently, many types of EPS produced by submerged cultures of mushrooms have been studied and are currently used for pharmaceutical purposes due to their diverse biological activities [12, 14-18]. Thus, the influence of culture conditions for improving the production of medicinal EPS from some mushrooms or enthomopathogenic fungi has recently been reported [8, 11, 19-21]. Although many studies have examined the effect of culture conditions on the production of microbial polysaccharides [22-24], little is known about the influence on the product quality, particularly

Corresponding Author: Dr. Tarek A.A. Moussa, Department of Botany, Faculty of Science, University of Cairo, Giza 12613, Egypt

molecular properties [25]. Industrial applications of polysaccharides have relied mainly upon raw materials from plants and marine algae until recently [26].

The worldwide food industry uses 70,000 tonnes of polysaccharides per year as thickening agents, stabilizers and texturisers. As the emerging food products become more complex and diverse, the requirement for new and versatile additives is stronger. Nowadays, different polysaccharides are used to modify food viscosity and texture. Additionally, polysaccharide gums constitute non-fat alternatives that may serve as a source of soluble dietary fibre with health beneficial effects at quite low levels. They are currently obtained from plants (starch, cellulose, pectin, guar gum), seaweed and crustaceans (alginate, carrageenans, chitosan) or microbial sources (xanthan gum) but the exploration for novel candidates still continues [27, 28].

The morphology of filamentous microorganisms in submerged culture has been shown to play a critical role in industrial fermentation and in commercial production of some metabolites [22, 29]. During submerged growth, many filamentous fungi can grow in either free mycelia or in pellets and their growth form is determined by a number of factors, one of which is shear effect [30]. Filamentous microorganisms are more sensitive to shear force in stirred-tank reactors than in other culture systems. A low rate of agitation is required to reduce the detrimental shear effect. However, most industrial fungal cultures are highly viscous and demand high concentrations of oxygen for acceptable product concentrations. Essentially, to satisfy adequate mixing and aeration, vigorous agitation is required [31].

The aim of this study, is to isolate and characterize the exodisaacharide (EDS) produced by *Penicillium chrysogenum*, as a better understanding of the chemical nature of this substance, chemical analyses such as ^{1}HNMR, FT-IR, MS, TGA and elemental analysis were performed to elucidate the structure of the produced ES. Also some growth parameters were tested.

MATERIALS AND METHODS

Isolation of the fungus and cultural conditions: During the course of investigation of soil micro-fungi in salt marsh locality of Red Sea coast located at about 60 km south Hurghada (c. 460 km south of Suez) in mangrove (*Avicennia marina*) area. One of the isolated *Penicillium* species showed some pale yellow secretion around and at the edge of colonies and appeared as prism-like crystals under the microscope. The fungus was cultivated in Dox's liquid medium to separate and isolate these crystals in pure form.

The fungus was identified according to Pitt [32] and Moubasher [33]. This identification was confirmed at Assiut University Mycological Center (AUMC) and graphed by transmission electron microscope at the Assuit University Electron Microscope Unit. The fungus maintained on a medium described by Johnson and Curl [34] supplemented with yeast extract.

Shake-flask cultures: Shake-flask culturing was performed in a 250 ml Erlenmeyer flask, which contained 100 ml of the broth medium and inoculated with 10 mm fungal disc from the margin. Flasks were incubated at 28°C for 10 days using an orbital incubator SI 50 (Stuart Scientific, UK) at 150 rpm.

Determination of mycelial dry biomass, protein and carbohydrate contents: Dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying overnight at 70°C to a constant weight. Proteins were estimated by the Folin–Ciocalteau phenol reagent method [35] using bovine serum albumin (Sigma) as a standard. The carbohydrate contents were tested quantitatively by the phenol-sulfuric acid method [36] with glucose as a standard. The absorbance was measured at a wavelength of 490 nm using a Spectronic 20 spectrophotometer.

Isolation and recovery of exodisaccharide (EDS): The EDS was isolated from the shake-flask cultures. The filtrate was added to four volumes of 95% propanol to precipitate the crude EDS. To facilitate the precipitation, the filtrates were maintained at 4°C for a further 12 h. The precipitated EDS was collected by centrifugation at 4000 rpm (Beckman Coulter Inc., Fullerton, CA, USA) for 15 min at 4°C; the supernatant was discarded. The precipitate was then re-suspended in an equal volume of 75% ethanol and centrifuged again as above. The precipitated disaccharide was dried at 60°C to remove residual ethanol. Finally, the residue was dissolved in an equal volume of distilled water, lyophilized and the weight of EDS was estimated.

Nuclear magnetic resonance spectroscopy (^{1}H NMR): The ^{1}H NMR chemical shifts were assigned using Joel ECA500 with Delta-2NMR spectrometer. Two mg of EDS was dissolved in 0.7 ml Dimethyl sulfoxide-Trifluoroacetic acid (DMSO-TFA) and spectra were recorded at 25°C in 5 mm tube.

Forior-Transformed Infra Red Spectroscopy (FT-IR): FT-IR spectroscopy was performed at Micro-Analytical Centre, Faculty of Science, Cairo University, using a Bruker Vector 22 Model FT-IR Spectrometer on 2 mg of freeze-dried EDS in 300 mg of KBr. Scans

were conducted with a resolution of 2 cm^{-1} in the range 4000-400 cm^{-1}.

Mass Spectroscopy (MS): Mass spectroscopy for the purified EDS was carried out using Jeol JMS-AX500 Mass Spectrometer at Central Laboratories, National Research Centre, the conditions of analysis were temperature 50-150°C at the rate 8°C for 20 min.

Thermogravimetric Analysis (TGA): Thermogravimetric analysis of EDS isolated from culture filtrate of *P. chrysogenum* was carried out sing a TGA-50 (Shmadzu, Japan) thermal analyzer under N_2 atmosphere. A heating rate of 10°C/min from 22 to 600°C was employed to investigate the thermal behaviour of the EDS.

Elemental analysis of EDS: The percentage of individual elements in the EDS produced by *P. chrysogenum* was measured at Micro-Analytical Center, Faculty of Science, Cairo University.

RESULTS

Identification of microorganism: Colonies on Czapek's+yeast extract agar attaining 3.5 cm after 10 days at 25°C±2, commonly velvety, margin white, conidial area in green colour; exudates abundantly produced as numerous yellow droplets (Plate 1). Conidiophores smooth-walled; penicilli asymmetrical terverticillate, with the main axis and branches bearing 2-5 metulae (Plates 2-4). The transmission electron micrographs showed that the conidia of *P. chrysogenum*

Plate 1: Photograph showing the growth of *Penicilium chrysogenum* on Czapk's + yeast extract

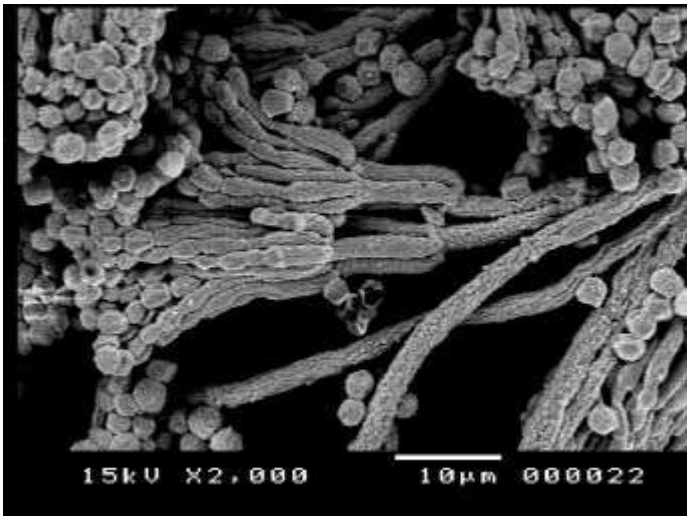

Plate 2: SEM micrograph showing conidiophores and chains of conidia of *Penecillium chrysogenum* (x= 2000)

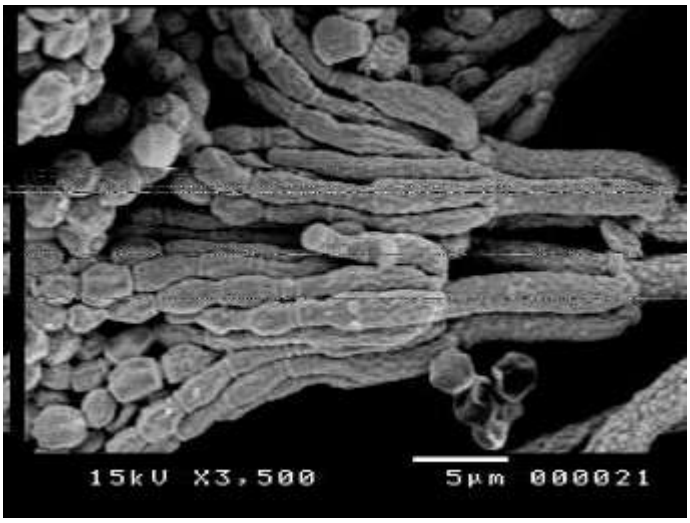

Plate 3: SEM micrograph showing conidiophores and chains of conidia of *Penecillium chrysogenum* (x= 3500)

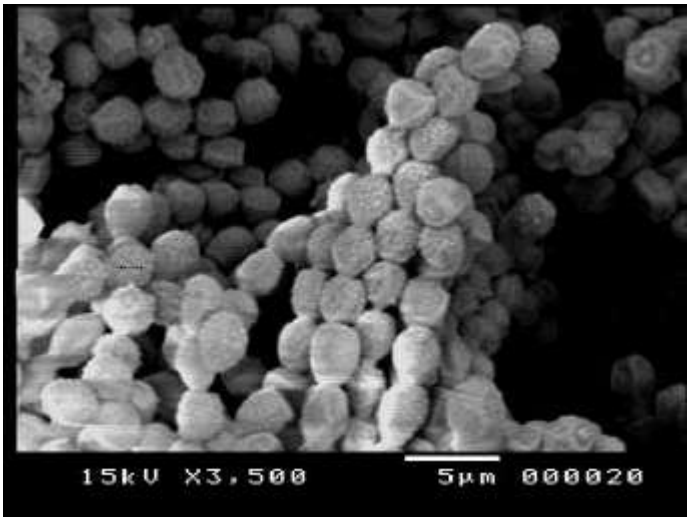

Plate 4: SEM micrograph showing chains of conidia of *Penecillium chrysogenum* (x= 3500)

are subglobose to ellipsoidal, 2.5-3.6 x 2.9-3.8 µm (Plates 2-4), smooth-walled, produced in long irregular columns.

Measurement of dry weight, protein content and carbohydrate content in culture filtrate: The dry biomass of the fungal mycelium was showed in Table 1. The biomass increases with increasing the time of incubation, where after 5 days incubation period the biomass was 8.0 g/l while after 10 days was 15 g/l. It is logic that the protein content in culture filtrate was increased with increasing the incubation time, this is due to production of hydrolyzing enzymes to absorb nutrients. After 5 days the protein content was 24.3 mg/ml culture filtrate, while after 10 days was 44.9 mg/ml. On the contrary, carbohydrate content of the culture filtrate was decreased with increasing the incubation time, due to absorption of them. The carbohydrate content after 5 days was 11.4 mg glucose/ml, while after 10 days was significantly

Table 1: Some parameters measured in the culture medium after growth of *Penicillum chrysogenum*

	Incubation period (days)	
Parameter	5	10
Dry biomass (g l^{-1})	8.00±0.05	15.00±0.45
Protein content (mg ml^{-1})	24.30±0.04	44.90±0.10
Reducing sugar (mg ml^{-1})	11.40±0.01	10.40±0.06
EDS concentration (g l^{-1})	0.60	2.50
Yield (g biomass/g reducing sugars)	0.70	1.44
Maximum EDS/biomass (g g^{-1})	0.08	0.17

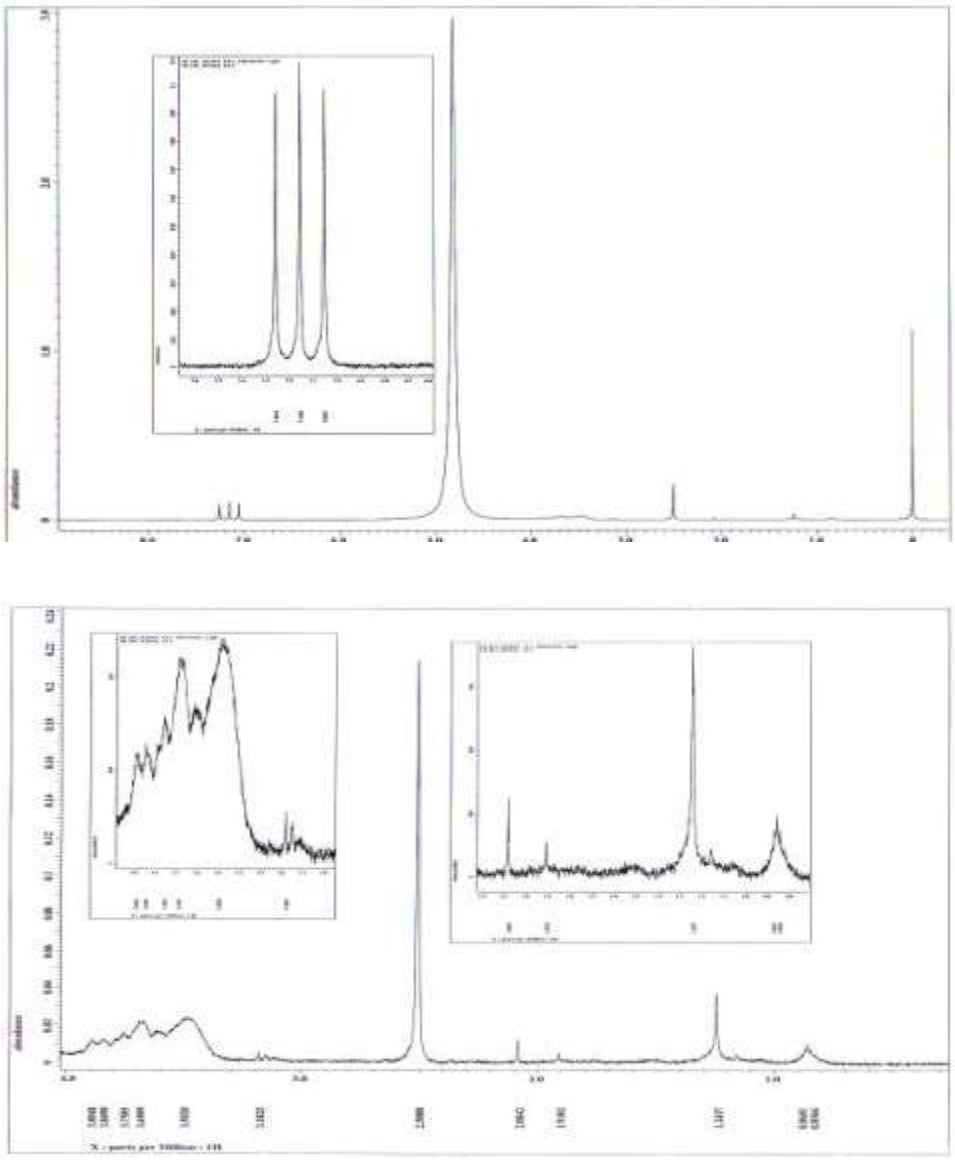

Fig. 1: [1]H NMR spectra of the EDS produced by *Penicillum chrysogenum*

decreased to 10.4 mg glucose/ml. The amount of EDS produced by *P. chrysogenum* after 5 and 10 days of cultivation periods (0.6 and 2.5 g/l, respectively). By calculating the yield (g of biomass/g of reducing sugars) was obtained after 5 days (0.7 g/g) and 10 days (1.44 g/g), also by calculating the maximum EDS/biomass (g/g) ratio, it is clear that after 10 days incubation period provides the best results, 0.17 g/g which is 2.13 times higher than in case after 5 days (0.08 g/g).

^{1}H NMR spectra: The ^{1}HNMR spectra of the EDS extracted from *P. chrysogenum* is shown in Fig. 1. The spectrum is well representation of protons from gycosidic groups of carbohydrates. ^{1}H chemical shifts in the anomeric region are observed at 4-6 ppm. A doublet in the region 4.9-5.6 ppm corresponding to ∝-configuration. A doublet at 4.1 and 4.5 ppm corresponding to ß-configuration was not observed. The chemical shifts from 3.502 to 3.895 ppm were assigned to protons of carbons C2 to C6 of the glycosidic ring. The signal at 5.261 and 1.244 ppm were assigned to the anomeric and methyl protons at position-6, respectively, of ∝-L-rhamnopyranosyl residues; also the signals at 3.895, 3.759 and 3.508 ppm were assigned to H_2, H_5 and H_4, respectively, of ∝-L-rhamnopyranosyl residues. The methyl and methylene protons are also observed at 0.857 and 1.244 ppm,

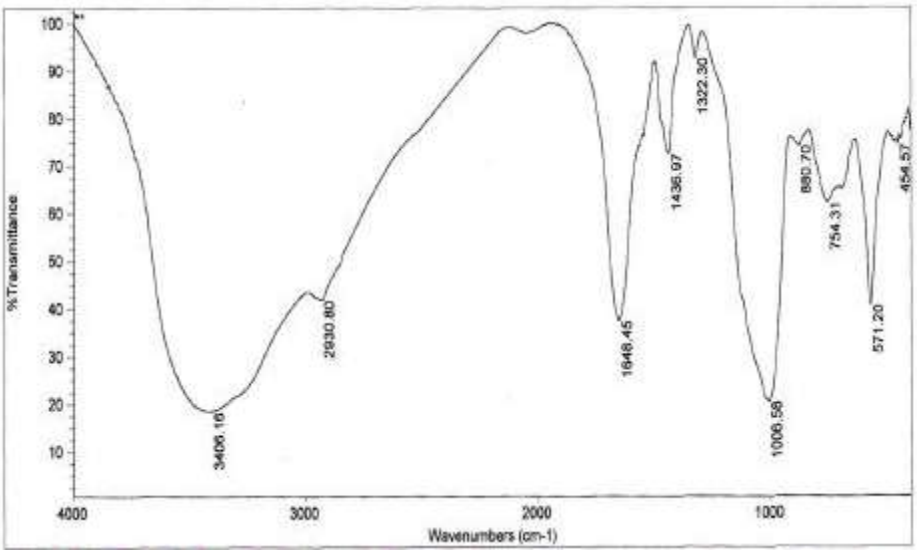

Fig. 2: FT-IR spectra of the EDS produced by *P. chrysogenum*

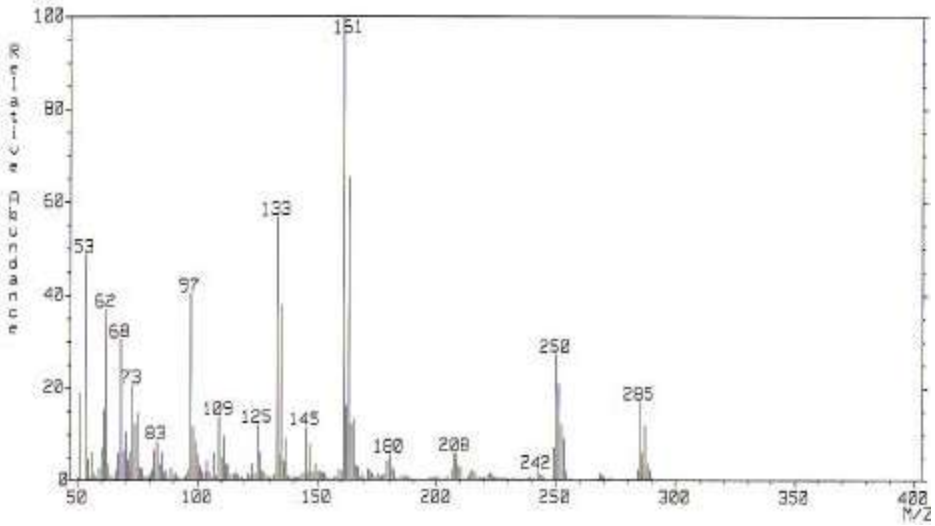

Fig. 3: Mass spectrum of the EDS produced by *P. chrysogenum*

respectively. The chemical shift at 2.78 ppm assigned to protein groups and the groups N-CH_3 and N-H which generally observed at 0.5 and 3.0 ppm, respectively, were not observed in this figure which indicates no protein residues in the EDS sample.

Forior-Transformed Infra Red Spectroscopy (FTIR): The FT-IR spectrum obtained for the EDS produced by *Penicilium chrysogenum* is represented in Fig. 2. Spectrum is consistent with those of typical carbohydrates: 3406.16 cm^{-1} (ν O-H) broad strong band due to hydroxyl groups and residual moisture, 2930.8 cm^{-1} (ν C-H) small band from the vibration of methyne methylene groups, 880.70 cm^{-1} small band indicating the presence of gylcosidic linkages and finally, 1648.45 cm^{-1} band is characteristic to anion carbohydrates. In FT-IR spectra, the C=O band of the aldehydic group was not observed.

Mass spectroscopy: The MS fragmentation pattern for the isolated EDS is shown in Fig. 3. The *m/z* is at 289 with base peak 161 (relative abundance 100%), 133 (relative abundance 55%), 53 (relative abundance 50%).

Thermogravimetric analysis (TGA): Thermogravimetric analysis is a simple and accurate method for studying the decomposition pattern and the thermal

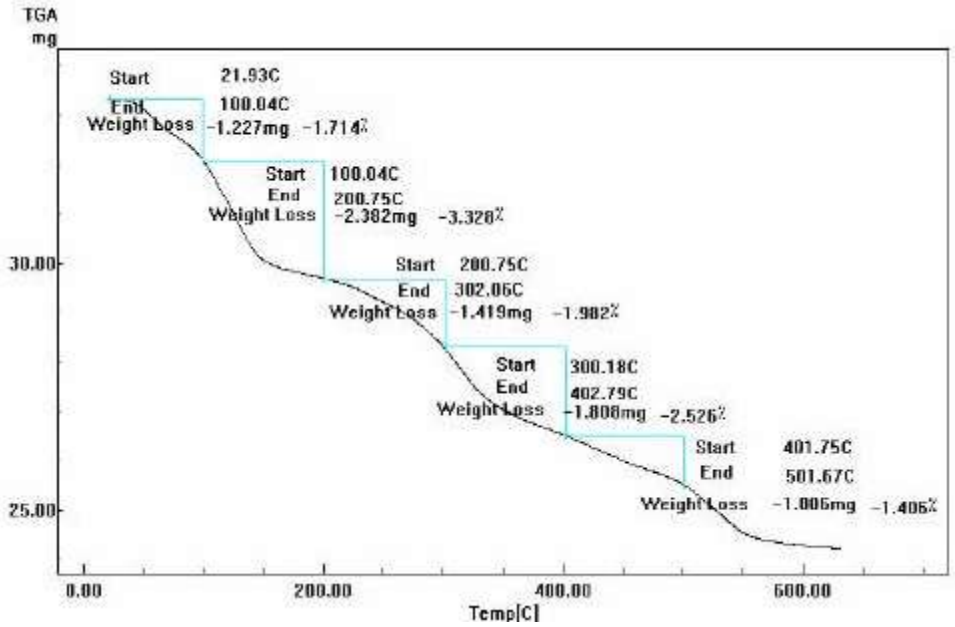

Fig. 4: Thermogravimetric analysis (TGA) of the EDS produced by *P. chrysogenum*

stability of polymers. Thermal gravimetric analysis of this disaccharide was also carried out. As it can be observed in Fig. 4, the EDS is thermally stable at all temperatures tested the temperature was increased by 100°C. After the 1st 100°C, the weight loss was 1.227 mg (1.714%), the weight loss was 2.382 mg (3.328%) after the 2nd 100°C, after the 3rd 100°C the weight loss was 1.419 mg (1.982%), after the 4th 100°C the weight loss was 1.808 mg (2.526%) and finally after the 5th 100°C the weight loss was 1.006 mg (1.406%). From the above we notice that the increase in temperature between 100°C-200°C a high weight loss compared to the other temperatures. Russo *et al*. [37] showed that alginates release water at different temperatures, depending on the different interactions of water with the polysaccharide.

DISCUSSION

According to Pitt [32] and Moubasher [33], the fungus was identified as *Penicillium chrysogenum* Thom. However, *P. chrysogenum* cannot be identified based on colour alone. Observations of morphology and microscopic features are needed to confirm its identity. *P. chrysogenum* has been used industrially to produce penicillin and xanthocillin X, to treat pulp mill waste, to produce the enzymes polyamine oxidase, phosphor-gluconate dehydrogenase and glucose oxidase [38].

The amount of EDS produced by *P. chrysogenum* was increased with increasing the incubation time. There are many contradictory results related to the effect of agitation and aeration on the yield of culture products and some confusion is often reported in literature as many culture variables can affect the fermentations. Whereas Wecker and Onken [39] suggested that highest pullulan yields were achieved at a combined low oxygen tension and low shear rate, where cell morphology was closely related to pullulan productivity. On the contrary, Gibbs and Seviour [22] demonstrated that pullulan production declined with increasing agitation rate.

Lee *et al*. [40] found that the culture pH, aeration rate and hydrodynamic behavior led to significant differences in the mycelial morphology of *G. frondosa* and subsequently affected EPS yields. Xu and Yun [41] found that aeration significantly affected the molecular characteristics of the EPS during submerged culture of an enthomopathogenic fungus *P. tenuipes* C240 in a stirred-tank fermenter. The results of Lin and Sung [42] indicated that nutrients can be utilized to improve the production of exopolysaccharide and that good mycelial growth does not seem to be a determining factor for a high production yield of exopolysaccharide in *A. cinnamomea*.

Wu *et al*. [31] showed that the EPS production was strongly associated with mycelial growth. Choa *et al*. [43] reviled that the maximum cell mass and EPS production were obtained at a relatively high agitation speed of 200 rpm and at an aeration rate of 2 vvm. Xu *et al*. [44] showed that molecular feature and chemical composition of the EPS are strongly dependent on the agitation intensity of bioreactors.

Moreover, agitation rates and associated shear force could attribute to variance in culture broth rheology and mycelial morphology, thereby affecting the productivities of mycelial biomass and EPS. It is likely that the effect of stirring speed on product quality of EPS is resulted from the bioreactor hydrodynamics (e.g., micromixing phenomenon) and the availability of oxygen. In conclusion, a combination of medium composition and environmental conditions should be carefully considered to control quality of EPS during the submerged mycelial culture processes of enthomopathogenic fungi.

The advantage of using NMR spectroscopy for structure elucidation of polysaccharides is that it does not involve uncertainaties resulting from chemical degradation and produces data for the intact polymer. The enhanced resolution capacity of high field NMR is definitely an advantage when analyzing large and rigid polysaccharides where broad signals and overlapping can be a problem. Our results were in agreement with Kawagishi *et al*. [45] who discussed that a doublet at 4.1 and 4.5 ppm is corresponding to the ß-configuration and in the region 4.9-5.6 ppm corresponding to ∝-configuration. Also, Chauveau *et al*. [46] deduced that the chemical shifts from 3.3 to 4.0 ppm were assigned to protons of carbons C2 to C6 of glycosidic ring. Silvestein *et al*. [47] concluded that the groups N-CH_3 and N-H are generally observed at 0.5-3.0 ppm, respectively.

Laurienzo *et al*. [48] found that the two proton peaks resonating at 3.36 (terminal O-CH_3) and 2.66 ppm (CH_2 at positions 2 and 3) in the PEG are observed upfield-shifted at 2.81 and 2.34 ppm, respectively, in AA-g-PEG. Furthermore, the methyl and methylene protons of the octyl chain are also upfield-shifted at 0.80 and 1.22 ppm, respectively. The anomeric proton region is also affected by the addition of PEG to alginate. In the AA–NHR, the anomeric protons are all clustered in the region 5.3-4.9 ppm, but upon addition of PEG they group in two well-separated regions at 5.47 and 4.97 ppm. They attributed such a spectral change to the formation of a hydrogen bond between the anomeric proton of the open alginate rings and the amidic carbonyl of the attached PEG, forming a stable six-membered ring.

Synytsya *et al*. [49] found that the FT-Raman and FT-IR spectra of polygalacturonic (pectic) acid, potassium pectate and their derivatives, as well as commercial citrus and sugar beet pectins were measured and interpreted. Methyl and acetyl esters of potassium pectate derivatives have several characteristic Raman and IR bands that allow both this groups to be distinguished. The very intense Raman band at 857 cm^{-1} is sensitive to the state of uronic carboxyls and to O-acetylation. The wavenumber of this band decreases with methylation (min. 850 cm^{-1}) and increases with acetylation (max. 862 cm^{-1}). The acetylation of potassium pectate, as well as its acetylation together with methylation, causes drastic changes in the Raman spectra in the region below 700 cm^{-1}. Sugar beet pectin, but not citrus pectin, showed Raman bands at 1633 and 1602 cm^{-1} and IR band at 1518 cm^{-1}. All these bands rise from feruloyl groups and can be used for identification of pectins containing feruloyl groups.

Cerná *et al*. [50] showed that the FT-IR spectroscopy in the 1200-800 cm^{-1} wavenumber region can be a very reliable technique for food authentication of polysaccharide-based additives and be used for a quick screening of polysaccharides used as additives in foodstuffs.

Mass Spectrometry (MS) is a valuable tool for analysis of oligosaccharides. A number of different techniques have been advocated as they often contribute different information and provide overlapping coverage for structural determination [51].

Laurienzo *et al*. [48] stated that three different kinds of interactions of water can be identified in the case of plain alginate: the first one, is free water that is released in the 40-60°C region; the second one, in the region 80-120°C, is water linked through hydrogen bonds and finally, water more tightly linked through polar interactions with carboxylate groups is released up to 160°C. The polymer starts to degrade around 200°C.

Zohuriaan and Shokrolahi [52] found that the early minor weight loss in samples is attributed to desorption of moisture as hydrogen bound water to the saccharide structure. The main decomposition of the polysaccharides starts above 200°C. However, the temperature at which methylcellulose starts to decompose is exceptionally high (325°C). For instance, while methylcellulose shows the highest initial decomposition temperature, it exhibits a fast degradation to yield very low char residue at 600°C (8%). In contrast, chitosan, CMC and gum tragacanth start to decompose slowly at 250–280°C and produce high char (20-35%) at the final temperature.

From the above chemical analyses like NMR, MS, FT-IR and TGA, the produced exo-disaccharide was composed from two molecules of L-rhamnose, which linked to each other by ∝-1,3 linkage. L-Rha*p*-∝-1,3-L-Rha*p*

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