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Pectinase enzyme-complex production by *Aspergillus* spp. in solid-state fermentation: A comparative study

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ABSTRACT

A comparative evaluation of three *Aspergillus* species according to their pectinase production in solid-state fermentation was performed. Solid-state fermentation offers several potential advantages for enzyme production by fungal strains. Utilization of agricultural by-products as low-cost substrates for microbial enzyme production resulted in an economical and promising process. The pectinolytic enzyme activities of two *Aspergillus sojae* strains were compared to a known producer, *Aspergillus niger* IMI 91881, and to *A. sojae* ATCC 20235, which was re-classified as *Aspergillus oryzae*. Evaluation of polymethylgalacturonase and polygalacturonase activity was performed as well as exo- vs. endo-enzyme activity in the crude pectinase enzyme-complex of the mentioned strains. Furthermore, a plate diffusion assay was applied to determine the presence and action of proteases in the crude extracts. *A. sojae* ATCC 20235 with highest polymethylgalacturonase activity and highest polygalacturonase activity both exo- and endo-enzyme activity, is a promising candidate for industrial pectinase production, a group of enzymes with high commercial value, in solid-state fermentation processes. Beside the enzymatic assays a protein profile of each strain is given by SDS-PAGE electrophoresis and in addition species-specific zymograms for pectinolytic enzymes were observed, revealing the differences in protein pattern of the *A. sojae* strains to the re-classified *A. oryzae*.
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Keywords: *Aspergillus sojae*; Pectinase; Polygalacturonase; Solid-state fermentation

1. Introduction

The middle lamella and the primary cell wall of higher plants contain a complex heteropolysaccharide called pectin. These carbohydrate polymers support the cohesion of the other cell wall polysaccharides and proteins. Pectin is composed mainly of galacturonic acid residues (Willats et al., 2001). Pectinases include a number of related enzymes involved in the breaking down of pectic substances. Therefore, they can cause plant tissue maceration, cell lysis, and modification of cell wall structures, allowing other depolymerases to further degrade their product of decomposition (Collmer et al., 1988). Pectinolytic enzymes are extensively used in the food industry, e.g., as processing aids, the largest industrial application being in fruit juice extraction and clarification. Break down of pectin reduces the viscosity of pectin-rich crude juice and thus increases juice flow and reduces the press-time. Pectinases are also involved in clarification of wine, oil extraction, removal of citric fruit peels, and degumming fibres (Jayani et al., 2005; Mutlu et al., 1999; Silva et al., 2002). Depolymerizing enzymes like polygalacturonases are distinguished according to their substrate preference, whether they have preference for poly[α(1 → 4)-D-methylgalacturonic acid] (pectin-like substrates), which are termed as PMG in this study or poly[α(1 → 4)-D-galacturonic acid] (pectic acid-like substrates), which are termed as PG (Whitaker, 1984). Furthermore these enzymes are termed as exo- or endo-enzymes depending on the action pattern. Endo-PGs randomly attack the [1 → 4]α-glycosidic linkages of the polysaccharide chain producing a number of galacturonic acid oligomers, while exo-PGs specifically hydrolyses at the non-reducing end of polygalacturonic acid. Commercial pectic enzymes used in food industry normally contain a mixture of enzymes that split pectic compounds; traditionally mixtures consist of PG,

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PL (pectin lyase) and PME (pectin methylesterase), and are associated with cellulytic, proteolytic and other species of enzymes apart from the main pectinases (Del Cañizo et al., 1994). In some food processes, it is convenient to use only one type of pectinolytic enzymes, e.g., preparation of instant potato flakes and carrot juice for baby food requires the maceration, where vitamins, color and aroma have to be preserved and for these applications preparations that mainly contain PG activity are preferred (Lang and Dörnenburg, 2000).

Filamentous fungi especially *Aspergillus niger* (*A. niger*), are the major producers of acidic pectic enzymes used in fruit juice industries and wine production (Kashyap et al., 2001; Naidu and Panda, 1998). Products of *A. niger* as well as *Aspergillus sojae* and *Aspergillus oryzae* have obtained a GRAS (General Regarded As Safe) status, which has approved their use in the food industry. Usually pectolytic microorganisms produce a multiplicity of pectinolytic enzymes. The production of these enzymes is carried out in solid-state (SSF) and submerged fermentation (SmF).

The utilization of SSF processes is interesting for pectinase production by fungi, because of its capability to grow in low water activity (a_w), which is a dimensionless quantity used to estimate the amount of free water that is readily available for the microorganisms. This SSF process offers several potential advantages in comparison to SmF, e.g., higher product concentration, simpler fermentation technology, and reduced waste-water output (Pandey et al., 2000). SSF also holds a tremendous potential for enzyme production. In several comparative studies on fungal pectinases production in solid-state and submerged fermentation, SSF gave superior results compared to submerged conditions and the protease production was also extremely lower (Díaz-Godínez et al., 2001; Favela-Torres et al., 2006; Patil and Dayanand, 2006a). Moreover, simple and economic agricultural by-products like wheat bran and orange peel could be utilized so as to provide both nutritional and physical support during solid substrate cultivation. Wheat bran, which is composed predominantly of non-starch carbohydrates like arabinoxylans or cellulose, starch and crude proteins, has been a preferred substrate for the production of pectinolytic enzymes (Sun et al., 2008).

Previous attempts to produce the pectinolytic enzyme polygalacturonase (PG) by *A. sojae* ATCC 20235 have included submerged and surface cultivation (Tari et al., 2007; Ustok et al., 2007). These studies have demonstrated the presence of enzymes with exo-PG activity in the crude (or partially purified) fermentation broths. These studies have triggered an interest in understanding the potential of *A. sojae* for the production of PG, as well as an eagerness of knowledge on the characteristics and technical applications of pectinases. On the other hand, SSF has been successfully employed for the production of the pectinase-complex from other *Aspergillus* strains, notably *A. niger* (Favela-Torres et al., 2006; Pandey et al., 2000).

The aim of this study focused on the comparative evaluation of three *Aspergillus* species including *A. niger* IMI 91881, as a known producer, *A. sojae* ATCC 20235, which was reclassified as *A. oryzae* (Heerikhuisen et al., 2005), *A. sojae* CBS 100928, and *A. sojae* IMI 191303 for the production of pectinolytic enzymes. This general screening was performed in order to identify potential pectinase producers. The focus of this work was the evaluation of pectinase production of exo- vs. endo-PG activity, as well as exo- vs. endo-polymethylgalacturonase (PMG) activity in the crude extract obtained in SSF of the mentioned strains. To the best of our knowledge, there is no available information in the open literature related to the production of both pectinases by the two *A. sojae* strains employed in this study.

2. Materials and methods

2.1. Materials

All chemicals were purchased from AppliChem GmbH (Darmstadt, Germany), except $FeSO_4{\cdot}7H_2O$ and polyvinylpyrrolidone 360 which were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Microbial substrates like wheat bran, orange peel, and molasses were obtained from local suppliers (Bremer Rolandmühle Erling GmbH & Co. KG, Bremen, Germany; Freeze-Dry Foods GmbH, Greven, Germany; Golden Sweet, Meckenheim, Germany). Substrates for detection of pectinolytic activities, e.g., pectin, polygalacturonic acid and polygalacturonic acid sodium salt were purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany.

2.2. Microbial strains and propagation

The *Aspergillus* strains used throughout this study were all purchased in lyophilized form from different culture collections and propagated on agar plates according to the specifications given by the culture collections. *A. sojae* ATCC 20235 was obtained from Procochem Inc. (Teddington, United Kingdom), an international distributor of the American Type of Culture Collection (ATCC) in Europe. Yeast Malt Extract (YME) agar medium, containing malt extract (10 g/L), yeast extract (4 g/L), glucose (4 g/L) and agar (20 g/L), was used for the propagation of this culture. Plates were incubated at 30 °C until abundant sporulation (1 week). It has to be noted that *A. sojae* ATCC 20235, which is still deposited as *A. sojae* at the ATCC, did not meet the requirements to be classified as *A. sojae* on the basis of morphological parameters (Ushijima et al., 1982) and has been recently reclassified as *A. oryzae* based on the *alpA* restriction fragment length polymorphism (RFLP) (Heerikhuisen et al., 2005).

A. sojae CBS 100928 was purchased from the Centraalbureau voor Schimmelcultures (CBS) (Utrecht, Netherlands). The propagation of this culture was done on Malt Extract Agar (MEA) plate medium containing, malt extract (20 g/L), peptone (1 g/L), glucose (20 g/L) and agar (15 g/L), and incubated at room temperature (22 °C) in darkness until sporulation (1 week).

A. sojae IMI 191303 and *A. niger* IMI 91881 were obtained from CABI Bioscience/United Kingdom National Culture Collection (UKNCC) (Egham, United Kingdom). The propagation of these cultures was done on Czapek agar plate medium containing per liter: sucrose (30 g), K_2HPO_4 (1 g), agar (15 g), and Capek concentrate (10 mL) containing per 100 mL: $NaNO_3$ (30 g), KCl (0.5 g), $MgSO_4{\cdot}7H_2O$ (0.5 g) and $FeSO_4{\cdot}7H_2O$ (0.01 g). The cultures were incubated at room temperature for 1 week until sporulation.

Stock cultures from spores of all mentioned strains were preserved in 20% glycerol and stored at −80 °C.

2.3. Inoculum

A pre-activation step was performed on agar plates, containing the mentioned medium (see Section 2.2) for propagation for each culture, using the stock cultures. Spores from these plates were used as inoculum for molasses slants. The spores for inoculation of the main culture were extracted from

molasses agar slants containing: glycerol (45 g/L), molasses (45 g/L), peptone (18 g/L), NaCl (5 g/L), KCl (0.5 g/L), $FeSO_4{\cdot}7H_2O$ (15 mg/L), KH_2PO_4 (60 mg/L), $MgSO_4$ (50 mg/L), $CuSO_4{\cdot}5H_2O$ (12 mg/L), $MnSO_4{\cdot}H_2O$ (15 mg/L) and agar (20 g/L). Spores were harvested from the slants using 5 mL of 0.02% (w/v) Tween 80 and counted microscopically.

2.4. Culture medium and growth conditions

Erlenmeyer flasks (250 mL) containing 10 g of wheat bran and fine-particle granulate of dried orange peel in the ratio 70:30, wetted at 120% with 0.2 N HCl solution (sterilized at 121 °C for 20 min) were inoculated with the spore suspensions and incubated at room temperature for 1–6 days. Each Erlenmeyer flask (250 mL) was inoculated with the total number of 2×10^7 spores.

2.5. Enzyme leaching

At each 24 h interval of cultivation, the enzyme recovery was obtained by adding 100 mL distilled water into the Erlenmeyer flask of each strain and mixed in an incubator shaker (Innova 4230, New Brunswick Scientific) at 350 rpm, 30 °C, for 30 min. The mycelium and solid medium were separated by filtration through cheese cloth and the filtrate was centrifuged at 4 °C, $3200 \times g$, for 20 min. Enzyme activities, pH, soluble carbohydrate from crude extract and total protein concentration were determined in the supernatant.

2.6. Analytical methods

2.6.1. Total protein determination

Total extracellular protein was measured according to the modified Bradford's method, using Coomassie Plus™ Protein Assay Kit (Pierce, Fischer scientific, Schwerte, Germany). The assay was performed in a microplate by determining the absorbance at 595 nm using bovine serum albumin (BSA) as a standard. Determinations were performed in duplicate.

2.6.2. Exo-pectinolytic activity measurement

2.6.2.1. Polymethylgalacturonase assay. PMG activity was determined by measuring the release of reducing groups from citrus pectin using a modification of the DNS method (Miller, 1959). Galacturonic acid was used as standard. The reaction mixture contained an appropriate dilution of the supernatant along with 0.5% (w/v) pectin dissolved in 0.1 M acetate buffer (pH 5.0), which was incubated at 45 °C for 10 min according to Blandino et al. (2002). One unit of exo-enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol of galacturonic acid per unit volume of supernatant per unit time at standard assay conditions. Enzyme activity was expressed as unit per gram dry substrate (U/g). Determinations were performed in duplicate.

2.6.2.2. Polygalacturonase assay. PG activity was assayed according to the Nelson-Somogyi method adapted by Panda et al. (1999), using 2.4 g/L polygalacturonic acid as substrate at pH 4.0. Crude extract (0.086 mL) containing enzyme was added to 0.4 mL substrate and incubated for 20 min at 26 °C. The corresponding galacturonic acid content was determined from the standard galacturonic calibration curve. As to the assay conditions mentioned above, one unit of exo-enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol of galacturonic acid per unit volume of supernatant per unit time at standard assay conditions. Enzyme activity was expressed as unit per gram dry substrate (U/g). Determinations were performed in duplicate.

2.6.3. Endo-pectinolytic activity measurement

Endo-enzyme activity was determined by measuring the decrease in viscosity of a substrate solution, either 2% (w/v) pectin for endo-PMG or 3.2% (w/v) polygalacturonic acid (sodium salt) for endo-PG. Pectinolytic activity was assayed by adding 0.2 mL of a 1/20 dilution of crude extract containing the enzyme, to 0.2 mL of 0.2 M acetate buffer (pH 5.0) and 1.6 mL substrate. The mixture was incubated in a water bath for 1 h at 40 °C with shaking. After incubation, the viscosity of the samples was determined. The later was done indirectly by measuring the time required for the reaction mixture to elute through a 1.0 mL glass pipette. Samples were measured in triplicate. Viscosity was calculated from time measurements to pass the standard solution through the pipette according to a calibration curve obtained utilizing PVP 360 standard solutions at 25 °C (Yeh et al., 1998). Controls for non-enzymatically treated substrate solutions were included, utilizing inactivated samples. One unit of Endo-PG activity was defined as the quantity of enzyme which caused a 50% reduction in viscosity of the reaction mixture per minute, under the conditions of the assay (Blandino et al., 2002).

2.6.4. Plate assay for proteolytic activity

Dual-substrate assay plates were prepared according to the procedure given by Montville (1983), containing 1% (w/v) casein and 1% (w/v) gelatin as substrates. Wells of 5 mm diameter were cut into the solid media and filled with 30 μL crude extract or commercial protease solution (as control). After 24 h incubation at 30 °C the diameters of the zones formed were measured. Zone diameters (D) were converted to $\log_{10}$ adjusted zone area by the following expression:

$$\log_{10}\text{adjusted zone area} = \log_{10}\left[\left(\frac{D}{2}\right)^2\pi - \left(\frac{5.0}{2}\right)^2\pi\right] \quad (1)$$

Proteolytic activity was reported in this manner and referred to as zone area ($\log_{10}$ mm^2). Determinations were performed in duplicate.

2.6.5. Total soluble carbohydrate assay

Soluble carbohydrates in crude extract were determined by the phenol–sulfuric acid method according to Dubois et al. (1955), using D-glucose as standard. For the assay, 1 mL of 5% (w/v) phenol solution and 5 mL 96% H_2SO_4 were added to 1 mL of an appropriate dilution of the supernatant. Samples were incubated at room temperature for 20 min. The absorbance of each sample was spectrophotometrically determined at 490 nm. Soluble carbohydrate content was expressed as mg per gram substrate.

2.6.6. Protein fractionation

2.6.6.1. One-dimensional electrophoresis. The supernatant of the crude extract obtained by SSF was first dialyzed over night at 4 °C, using SnakeSkin® pleated dialysis tubing, 3500 MWCO (Thermo Scientific, Rockford, USA). Samples were centrifuged at 4 °C, $6000 \times g$, for 20 min and concentrated 15 times, using a freeze-dryer. SDS-PAGE was performed according to Laemmli (1970). Briefly: 12.5% SDS-PAGE gels with an approximately 2 cm stacking buffer zone were cast and samples run in constant current mode at 20 mA/gel, at 4 °C. Samples were

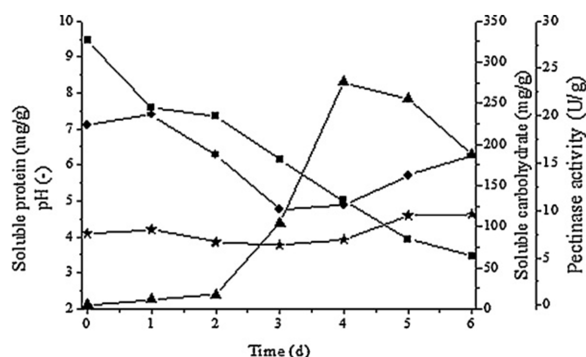

Fig. 1 – Cultivation profile of *A. niger* IMI 91881: (-▲-) PMG activity, (–■–) soluble carbohydrate content, (–♦–) soluble protein content and (–★–) pH in the crude extract.

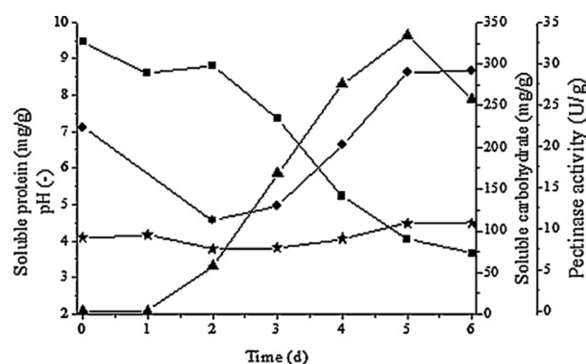

Fig. 2 – Cultivation profile of *A. sojae* ATCC 20235: (-▲-) PMG activity, (–■–) soluble carbohydrate content, (–♦–) soluble protein content and (–★–) pH in crude extract.

mixed with sample buffer in the ratio 2:1. Sample load add up to 10 μL/lane. Protein bands were visualized, using colloidal Coomassie (G-250) staining (Neuhoff et al., 1988).

2.6.6.2. Native polyacrylamide gel electrophoresis/zymogram. Native PAGE was performed by excluding SDS and DDT from the electrophoresis protocol described above. The "sandwich" method was used to detect the activity of pectinases acting on polygalacturonic acid as substrate (Manchenko, 1994). Briefly, proteins were separated and subsequently the gel was (a) incubated for 20 min in 0.1 M citrate phosphate buffer (pH 5) and (b) contacted with an (solid) agar substrate containing 0.25% (w/v) polygalacturonic acid for 80 min at 30 °C (80% humidity chamber). The polygalacturonic acid agar plate was treated with 1% (w/v) cetyltrimethylammonium bromide which revealed pectinases activity as translucent bands on an opaque background.

3. Results and discussion

3.1. Culture profiles on solid substrates

Solid substrate cultivation experiments were performed to evaluate the production of pectinases by several fungal strains which belong to the genus *Aspergillus*. The cultivation procedure for the different strains was performed under the same conditions, utilizing wheat bran moistened with a diluted hydrochloric acid solution (Fernandez Lahore et al., 1997). Grinded (dehydrated) orange peel was added to the solid substrate as an inducer for the production of pectinolytic enzymes (Patil and Dayanand, 2006b). Thus, the opportunity for a direct comparison between different *Aspergillus* strains in terms of pectinase production was set. Information of this kind is seldom available in the literature since most comparisons of pectinase activity between several strains are done under very different cultivation conditions and using different analytical assays to quantify the enzymatic activity (Favela-Torres et al., 2006; Naidu and Panda, 1998). Experiments were carried out in duplicate and the corresponding standard variation was below 15%.

3.1.1. Cultivation of A. niger IMI 91881

The cultivation profile of *A. niger* IMI 91881 is shown in Fig. 1. From the mentioned graph, the total soluble carbohydrate content, the total soluble protein content, the pH, and the PMG activity of the crude extract obtained from the fermented mass can be observed. The SSF process was run for six days. In this case, the peak of PMG activity production (∼23.5 U/g) was observed on the 4th day of cultivation. Hence, a space-time-yield of PMG production of 5.9 U/g/day can be calculated. The total protein content decreased at the beginning of the fermentation but from the 3rd day of cultivation the observed protein secretion was higher than the degradation of soluble proteins. The increasing protein concentration could indicate the production of extracellular enzymes. During the course of SSF, soluble carbohydrate were degraded from 320 mg/g to 60 mg/g indicating extensive utilization of the carbon and energy (C/E) source available for biomass and product synthesis. The pH in the crude extract evolved from 4.1 to 4.6 thus, confirming the appropriate selection of the acidic conditions to favor mold growth while avoiding bacterial contamination of the system. *A. niger* is well known for its ability to produce pectinases in both solid substrate and submerged fermentation systems (Minjares-Carranco et al., 1997). Pectinolytic enzymes from this species are industrially produced (Kester and Visser, 1990).

3.1.2. Cultivation of A. sojae ATCC 20235

Fig. 2 depicts the process parameters measured during the solid substrate cultivation of *A. sojae* ATCC 20235. This strain has been extensively studied before, particularly in relation to its ability to produce enzymes with exo-polygalacturonase activity in submerged and surface cultivation (Tari et al., 2007; Ustok et al., 2007; Gögus et al., 2006). When subjected to fermentation on solid substrates in the absence of free water, *A. sojae* ATCC 20235 produced a maximum PMG activity of 33.4 U/g at the 5th day of cultivation. Thus, the calculated space-time yield for PMG production amounts to 6.7 U/g/day. While PMG production was negligible during the early cultivation stage, it was observed that between the 2nd and 5th day of cultivation the PMG activity sharply increased. A similar trend was observed in the total protein content of the extracts; a higher secretion than degradation of soluble protein was noticed from the 2nd day of fermentation onwards. This could indicate that *A. sojae* ATCC 20235 is a good protein exporter under SSF conditions. Total soluble carbohydrate levels decreased abruptly after the 2nd day of cultivation indicating utilization for biomass and protein production. The pH of the extracts only increased slightly during the time course of the cultivation process.

3.1.3. Cultivation of A. sojae IMI 191303

The fermentation profile of *A. sojae* IMI 191303 on solid substrate is presented in Fig. 3. It can be observed that the peak of exo-PMG production (19.2 U/g) is reached on the 4th day of cul-

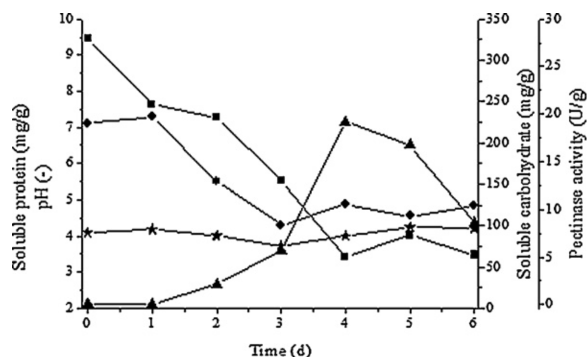

Fig. 3 – Cultivation profile of *A. sojae* IMI 191303: (-▲-) PMG activity, (–■–) soluble carbohydrate content, (–♦–) soluble protein content and (–⋆–) pH in crude extract.

tivation. This translates into a space-time yield of 4.8 U/g/day. While pectinolytic activity decreased after the 4th day of fermentation, the degradation of carbohydrates stagnated after that fermentation time, too. This would suggest that the product synthesis has been limited by the unavailability of the C/E source. Total protein was also degraded during the first 3 days of cultivation and remained at a relatively low level afterwards (~5 mg/g). The pH of the extracts varied only slightly and remained within acidic range, as observed in the case of the other strains mentioned before. Previous studies on pectinase production by *A. sojae* were done in the 1970s and focused on the enzyme activity of pectin transeliminase (Ishii and Yokotsuka, 1972). Recent publications about the strain *A. sojae* explore the production of recombinant proteins (Heerikhuisen et al., 2005). To the knowledge of the authors no other publications about pectinase production by *A. sojae* of other research groups are published.

3.1.4. Cultivation of *A. sojae* CBS 100928

The cultivation profile of *A. sojae* CBS 100928 is shown in Fig. 4 The production of enzymes with exo-PMG activity can be observed after the 2nd day of fermentation. However, maximum values are reached at the 3rd day (14 U/g) and the 5th day (16.6 U/g). This has coincided with a moderate increase in total protein concentration which was noticed after 4 days of cultivation. The presence of a plateau at the total protein concentration between 2 and 4 days incubation time – or eventually a bimodal activity profile – may indicate the presence of various types of enzymes presenting distinct synthesis kinetics. Considering the values found at the 5th day of cultivation, a space-time yield of 3.3 U/g/day for PMG production can be calculated. Comparing the two *A. sojae* strains a higher exo-PMG activity was observed by *A. sojae* IMI 191303 under the growth conditions used in this study. Total soluble carbohydrate content decreased gradually, particularly after the 3rd day of cultivation. The pH values measured in the extracts were comparable to the other observed cultivations reported here. As reported by Sardjono et al. (1998) *A. sojae* and *A. oryzae* are both producers of microbial proteases, this aspect is further explored in the next sections.

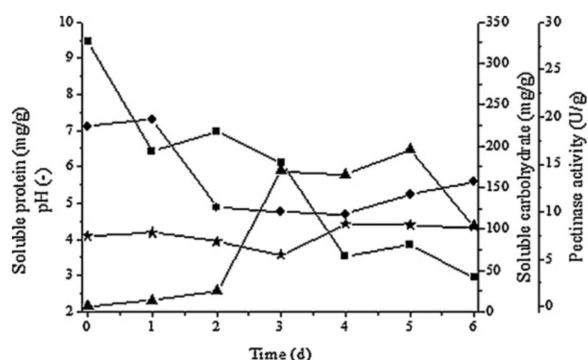

Fig. 4 – Cultivation profile of *A. sojae* CBS 100928: (-▲-) PMG activity, (–■–) soluble carbohydrate content, (–♦–) soluble protein content and (–⋆–) pH in crude extract.

3.2. Enzymatic activities of the crude extracts

The depolymerizing hydrolases of the pectinase group are distinguished according to their substrate preference and can be further subdivided depending on the action pattern as mentioned above. Usually the combined action of pectinolytic enzymes in combination with cellulytic, proteolytic and other species of enzymes is used in food industries. Following results reveal an overview of some enzymatic activities present in the crude extracts of the studied *Aspergillus* species.

3.2.1. Exo-PMG and exo-PG enzymatic activities

Table 1 compares the types of enzymatic activity found in the crude extracts obtained by harvesting the fermented mass of the various strains studied at the 5th day of cultivation. This time was fixed to standardize the cultivation conditions for a better comparison of all strains. At the 5th day of cultivation the peak of pectinase production or a plateau of enzyme production was observed. Hence, it was possible to assure high enzyme activity for all strains. It can be observed that all the cultures were able to produce enzymes with reducing-sugar liberation capability on both pectin (exo-PMG) and polygalacturonic acid (exo-PG). According to the exo-PMG potency, the mentioned extracts can be ordered as follows:

A. sojae ATCC 20235 > *A. niger* IMI 91881 > *A. sojae* IMI 191303 > *A. sojae* CBS 100928

While according to their exo-PG activity, the extracts can be ordered as follows:

A. sojae ATCC 20235 > *A. sojae* CBS 100928 > *A. niger* IMI 91881 > *A. sojae* IMI 191303

Interestingly, the total amount of protein found in the corresponding extracts follows a similar pattern, indicating that the total reducing-sugar liberating activity may be linked to the protein exportation capability of the strains under study. Moreover, the exo-PMG specific activity for *A. sojae* ATCC 20235 was higher (3.9 U/mg) than the observed for the other species, which may indicate that pectinases are a main component of the secreted proteome of the mentioned microorganism.

The results presented in Table 1 confirm previous findings by Ustok et al. (2007) who were able to produce exo-PG by *A. sojae* ATCC 20235 *via* surface cultivation on a complex media containing crushed maize, maize meal, corncob, and molasses broth. Since this strain was re-classified as *A. oryzae*, these results confirm previous findings by Malvessi and da Silveira (2004); they studied the production of exo-PG activity and endo-PMG activity by *A. oryzae* CCT3940 in a complex media based on wheat bran, salts, and pectin as an inducer in SmF. Utilizing this media resulted in a maximum exo-PG activity of 45 U/mL after 64 h and a maximum endo-PMG activity of 159 U/mL after 83 h of cultivation at 28 °C and 300 rpm. Furthermore, they observed that acidic initial pH values close to 4 favored both mycelia development and enzyme production. Comparing the endo-pectinase activity to different strains of *A. niger* resulted in higher activities for *A. oryzae* CCT3940. On

Table 1 – Enzyme activity at the 5th day of cultivation.

Microorganism	PMG-activity (U/g)		Ratio	PG-activity (U/g)		Ratio	Total protein (mg/g)	Specific activity (U/mg)	Protease activity ($\log_{10}$ mm^2)
	EXO	ENDO		EXO	ENDO			Exo-PMG	
A. niger IMI 91881	21.7	14.3	1.52	19.7	2.3	8.57	5.7	3.8	1.5
A. sojae ATCC 20235	33.4	32.9	1.02	28.3	30.1	0.94	8.6	3.9	2.4
A. sojae IMI 191303	16.7	4.8	3.48	14.9	18.8	0.79	4.6	3.6	1.8
A. sojae CBS 100928	16.6	28.8	0.57	23.0	23.0	1.00	5.2	3.2	2.1

the other hand, Galiotou-Panayotou et al. (1993) produced a maximum exo-PG activity of 14.5 U/mL by *A. niger* NRRL-364 (also known as *A. niger* IMI 91881) in submerged fermentation with a space-time yield of 4.8 U/mL/day. Employing this strain in SSF, an exo-PG activity of 19.7 U/g was observed after 5 days. According to the results of this study *A. niger* produced an extract characterized by the predominant presence of exo-pectinase activity, which implies the degradation of pectic substances into mono- or digalacturonic acid.

3.2.2. *Endo-PMG and endo-PG enzymatic activities*

The crude extracts obtained from the SSF processes employing various *Aspergillus* strains were assayed for endo-PMG and endo-PG activity. In this study, the ability of the enzymes present in the crude extract to reduce the viscosity of substrate solutions (2% pectin and 3.2% polygalacturonate, respectively) was observed. According to the results listed in Table 1, the endo-enzymatic potency of the extracts can be ordered as follows:

Endo-PMG:

A. sojae ATCC 20235 > *A. sojae* CBS 100928 > *A. niger* IMI 91881 > *A. sojae* IMI 191303

Endo-PG:

A. sojae ATCC 20235 > *A. sojae* CBS 100928 > *A. sojae* IMI 191303 > *A. niger* IMI 91881

The highest endo-pectinase activities where obtained by *A. sojae* ATCC 20235. Using a 1/20 dilution of this crude extract resulted in a viscosity reduction of 90.3% for pectate and 98.6% for pectin under the conditions mentioned above. In comparison to this, the commercial pectinase of *A. niger* (Sigma) had to be diluted 5000 times to reduce the viscosity of polygalacturonic acid by 90.4% and of pectin by 96.2% under the same conditions. To better understand the characteristics of the crude extracts the ratio PMG-exo-to-endo and the ratio PG-exo-to-endo were calculated. *A. sojae* ATCC 20235 showed a very well balanced ratio in both cases (~1), showing that the enzymes produced by this microorganism have a broad substrate action pattern. Besides that, the mentioned strain also provided the best production levels of pectinolytic enzymes, which promise an extensive degradation of pectic substances (Table 1). *A. sojae* CBS 100928 demonstrated to be a good producer of endo-pectinases. However, the PMG ratio for this strain was 0.57 while the PG ratio was 1. This indicates a preferential endo-PMG enzymatic activity. On the contrary, *A. sojae* IMI 191303 showed a remarkable PMG-ratio of 3.48, indicating a significantly higher exo-enzymatic activity, while on consideration of the PG-ratio (0.79) the endo-enzymatic activity is dominant. *A. niger* IMI 91881, as mentioned before, presented a marked exo-PMG activity (ratio 1.52) and extraordinary exo-PG activity (ratio 8.57) due to the low endo-enzymatic activity when acting on pectic substrates. Summarizing, it was shown that *A. sojae* ATCC 20235 produced a balanced pectinase-complex, while *A. niger* IMI 91881 produced preferential exo-pectinases and low endo-PG activity. *A. sojae* CBS 100928 delivered a predominant endo-PMG profile compared to the endo-PG activity, and *A. sojae* IMI 191303 delivered higher endo-enzymatic activity on polygalacturonate than on pectin.

However, since the effect on viscosity is dependent on location of the glycosidic bonds, and the hydrolysis of a glycosidic bond near the middle of the polymer chain has far more effect on viscosity reduction than hydrolysis near the end of the chain, the endo-enzymatic activities are the crucial factor for applications of viscosity reduction. Therefore endo-PGs have

especially been applied for maceration (Lang and Dörnenburg, 2000). Nevertheless, there is no direct correlation between viscosity reduction and number of glycosidic bonds hydrolyzed. Furthermore, Baker and Bruemmer (1972) demonstrated the effect of endo-depolymerizing enzymes to stabilize cloud in orange juice, at which the important fact was the ratio of high endo-PG activity to low endo-PMG activity. Concerning this, *A. sojae* IMI 191303 produced a preferable extract for such applications.

3.2.3. *Proteolytic activity*

The crude extracts were tested for total proteolytic activity. The presence of proteases in the extracts may render positive or negative consequences, depending upon the final application thought. As mentioned before, commercial pectinase mixtures also contain proteolytic enzymes (see Section 3.2), which is similar to the markets for microbial proteinases for the baking and leather industries, i.e., usually a processing aid in a proprietary blend with other ingredients is purchased, not just an enzyme (Outtrup, 1990). For example, mixtures with proteinases from *A. oryzae* are used for leather bating and blends containing fungal acidic proteinases are predominantly used for modification of high gluten doughs and for cracker and biscuit production in the bakery. On the other hand, protease-deficient strains are of use for the production of secreted recombinant proteins (Heerikhuisen et al., 2005). According to Table 1, the proteolytic potency of the extracts can be ordered as follows:

A. sojae ATCC 20235 > *A. sojae* CBS 100928 > *A. sojae* IMI 191303 > *A. niger* IMI 91881

A. niger IMI 91881 produced the lowest proteolytic activity, which could be an advantage regarding the stability of the enzyme concentrate e.g., protease attack on pectinases is less likely to occur or may occur in a lesser extent. The other three strains, however, showed a tendency to produce higher levels of proteases. *A. sojae* ATCC 20235 (re-classified as *A. oryzae*) appeared to be the strain producing a larger amount of extracellular protein, as well as, the higher levels of the enzyme types explored in this work. Therefore, an application of the SSF-derived extract to cases were extensive action on complex structures is required can be envisioned for this strain. Oda et al. (2006) demonstrated the ability of *A. oryzae* to secrete large amounts of a wide range of different enzymes into its environment and a higher extracellular protein production in SSF than under submerged culture conditions for this species.

3.3. *Protein fractionation studies*

3.3.1. *Fractionation by SDS PAGE electrophoresis*

Samples of the crude extracts obtained by extraction of the solid substrate cultures were analyzed by polyacrylamide-gel electrophoresis under denaturing conditions. Results are presented in Fig. 5. The observed protein patterns differed in complexity and the number of bands present in each species. Analysis of SDS-PAGE of whole-cell protein profiles has turned out as a useful tool for classification and identification of bacteria and fungal species (Bent, 1967; Merquior et al., 1994; Vancanneyt et al., 1992). According to the crude extracts obtained in this study there is an appropriate clear difference in the protein pattern of the *A. niger* strain, the *A. sojae* strain which was reclassified as *A. oryzae* and the two *A. sojae* strains. The extracts of *A. sojae* IMI 191303 and *A. sojae* CBS 100928 produced similar profiles. The profile of the commercial pectinase solution (SIGMA) presented three remarkable bands in

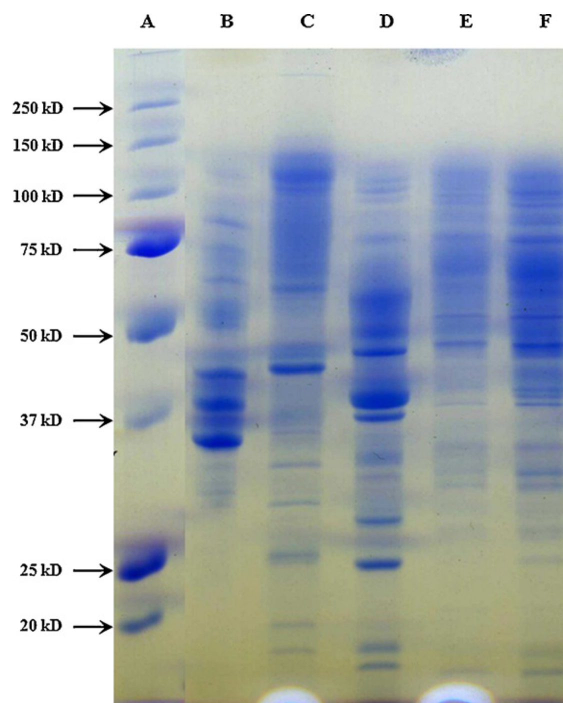

Fig. 5 – Analysis of the crude extract from the 5th day of SSF by SDS-PAGE: lane A: dual color prestained precision plus protein standards (BIO-RAD); lane B: commercial pectinase from *A. niger* (SIGMA) diluted 1:3; lane C: *A. niger* IMI 91881; lane D: *A. sojae* ATCC 20235; lane E: *A. sojae* IMI 191303; lane F: *A. sojae* CBS 100928.

the range 35–44 kDa and a number of thin bands with molecular masses in the range 50–120 kDa. Purifying pectinolytic enzymes from a commercial enzyme preparation derived from *A. niger* (Pectinase K2B 078, Rapidase) Kester and Visser (1990) isolated two most abundant endo-PGs with molecular masses of 55 and 38 kDa. The molecular weights of some members of the pectinases complex produced by *A. niger* in SSF have been determined (Dinu et al., 2007): PG (36 kDa), PME (42 kDa), PeL I (pectate lyase) (42 kDa), PeL II (30 kDa), PeL III (36 kDa).

3.3.2. *Native electrophoresis and zymogram*

The protein content of the extracts was also fractionated by native electrophoresis so as to preserve enzyme function. Results are shown in Fig. 6a. The polyacrylamide gel slab was subsequently contacted with a solid media surface that contained polygalacturonate as substrate. Fig. 6b depicts the results obtained after revealing the substrate plate chemically. It can be observed that the different *Aspergillus* species developed a distinctive pattern. Nasuno (1972) differentiated the species *A. sojae* and *A. oryzae* on the basis of species-specific mobility of alkaline proteinases in polyacrylamide gel disc electrophoresis under non-denaturing conditions. It has to be recalled that the active fractions (bands or zones) observed in this case are not directly related to the molecular mass of the enzymes since separation in the native gel is related to charge/size. This makes the information gathered in Fig. 6 (native PAGE) difficult to compare with the profiles observed in Fig. 5 (SDS-PAGE). Nevertheless, it can be clearly observed that *A. niger* presented a single active component, as is the case for *A. sojae* ATCC 20235, although both bands radically differed in electrophoretic mobility. While the commercial pectinase mixture (SIGMA) depicts at least 4 active zones, whereof one of them is outstanding. *A. sojae* IMI 191303 and *A. sojae* CBS

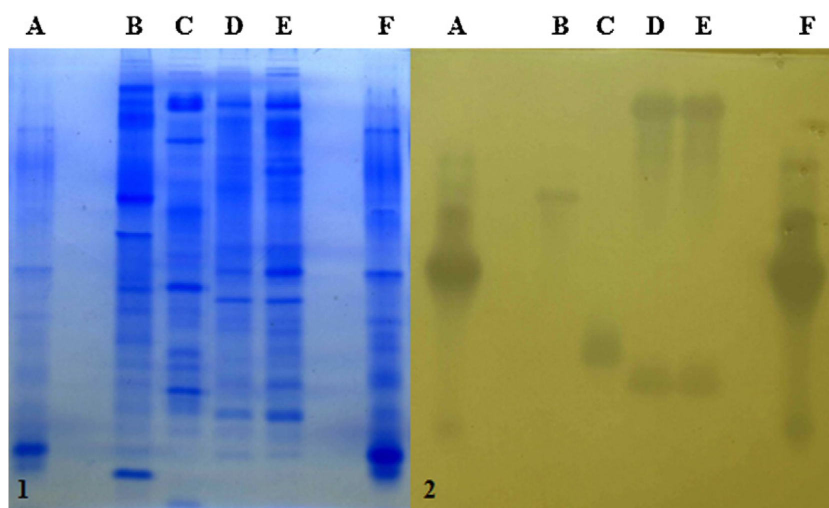

Fig. 6 – Native PAGE for enzyme detection on the electrophoretic gel. (1) Substrate-containing agar plate after precipitation; (2) electrophorized gel; lane A: commercial pectinase from *A. niger* (SIGMA) diluted 1:5; lane B: *A. niger* IMI 91881; lane C: *A. sojae* ATCC 20235; lane D: *A. sojae* IMI 191303; lane E: *A. sojae* CBS 100928; lane F: commercial pectinase from *A. niger* (SIGMA) diluted 1:6.

100928 presented noteworthy similar profiles: two main active zones well differentiated one from the other in terms of migration distances. Hence, similar to the SDS-PAGE profiles, the obtained zymograms for pectinolytic enzymes of the fungi were characteristic for each species like a kind of fingerprint to distinguish peculiar characteristics in the extracts of the used *Aspergillus* species. Nealson and Graber (1967) already distinguished species in the genus *Aspergillus* by means of esterase and phosphatase zymograms. Further studies are required to fully understand the nature and properties of the *A. sojae* extracellular enzymes.

4. Conclusion

This work demonstrated the use of three *Aspergillus* species for pectinolytic enzyme production in SSF. All strains produced pectinases with the highest yield reached between the fourth and fifth day of cultivation. Two new *A. sojae* strains were identified to express enzymes of this group. The zymogram for pectinolytic enzymes of that species presented two separated zones with activity towards polygalacturonic acid. Nevertheless, the highest exo-pectinolytic activity with 33.4 U/g PMG and 28.3 U/g PG, as well as the highest endo-enzyme activity of 32.9 U/g PMG and 30.1 U/g PG was observed by *A. sojae* ATCC 20235 (re-classified as *A. oryzae*). The high protein secretion in combination with the GRAS status of this strain seems to be promising with regard to enzyme production for industrial applications. Further optimization experiments using this strain for the production of pectinases may provide auspicious results in the future. Moreover, it was shown that the use of complex media in SSF also holds a great potential for the production of these enzymes. The yields might be greatly increased by manipulating some growth conditions. On the basis of this work further studies on the pectinase-complex obtained by *A. sojae* ATCC 20235 in SSF will follow.

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