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PECTINOLYTIC ACTIVITY OF *Boeremia strasseri* THE CAUSAL AGENT OF BLACK STEM AND RHIZOMES ROT OF PEPPERMINT

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Abstract. Pectinases have multiple nature and various forms which are necessary for hydrolysis of pectin in natural processes. The pectinolytic activity of three isolates of the pathogenic fungus *Boeremia strasseri* was demonstrated. The pectinases production was studied using a liquid Mandels-Weber medium, containing 1.0% citric pectin as a carbon source. Optimum pH and temperature for pectinases activity were 4.7 and 40°C respectively. The pectinases were totally stable at 40°C for 80 min. The greatest loss of activity was observed during the heating of the enzymes at 70°C.

Key words: *Boeremia strasseri,* pectinolytic activity, pectinases production, thermostability, fungi

INTRODUCTION

Among many metabolites of pathogens, especially fungi, active in the pathogenesis of plant, enzymes play a key role. Pathogens' enzymes involved in the disease process primarily act as catalysts in the reaction of decomposition of plant structures and thus they facilitate the process of infection and colonization of the host [Okafar et al. 2010]. In both processes, the most significant barriers to overcome are the cuticle, the cell wall and the middle lamella. The essential elements of the latter two structures are pectin compounds [Schols and Voragen 1996]. Pectins are complex heterogeneous polysaccharides. They are mainly composed of polyuronides linked together by α-(1,4)-glucosidic bonds of D-galacturonic acid-, for the most part esterified with methyl groups [Gummadi and Panda 2003]. The enzymes involved in the degradation of pectin are pectic lyase, pectin lyase and polygalacturonase which break α-(1,4)-glycosidic linkages to isolate pectin methyl ester group located at the C-6 and rhamnogalacturonase which are

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capable of binding to degrade α-(1 2) between galacturonic acid and rhamnose residues [Delgado et al. 1992, Mohamed et al. 2003].

Boeremia strasseri sp. nov. (*Phoma strasseri*) causes black stem and rhizomes rot of peppermint (*Mentha piperita* L.), also called phomosis of mint [Boerema et al. 2004, Zimowska 2007]. The disease symptoms on the plants of peppermint are visible on the stems, first in the form of necrotic, slightly hollow spots enfolding the stem around up to 10 cm from the base. With time, the tissue in the place of the spots gets rotten. The rot proceeds very fast on the rhizomes. Young rhizomes rot away wholly, while the bark layer often comes off on older ones [Horner 1971, Zimowska 2007]. From the literature it is known that enzyme β-glucosidase [Melouk and Horner 1973] participates in the process of pathogenesis of *B. strasseri* which catalyses the decomposition reactions of cellobiose to glucose and pectolytic enzymes breakdown of pectin to low molecular weight compounds, which are the primary source of carbon for the pathogen [Melouk and Horner 1972]. Melouk and Horner [1973] showed that the optimum pH for the activity of β-glucosidase is within the range of 4.5–5.5. No information is found in the available literature on the characteristics of pectinolytic enzymes produced by *B. strasseri.* Accordingly, the present work undertakes studies indicated in the title, and thermostability of enzymes produced by the three isolates obtained by the authors is determined.

MATERIALS AND METHODS

Fungal isolates. The studies used one-spore cultures of three isolates of *B. strasseri* (M 126, M 289 and M 743) obtained from the naturally infected plants of peppermint with the symptoms of black stem and rhizomes rot from the production plantations situated in the south-eastern part of Poland [Zimowska 2007]. Isolates included in the study had been previously tested for pathogenicity, according to Koch's postulates and all of them obtained high values of the infection index [Zimowska 2012]. Isolates of *B. strasseri* were incubated on a maltose agar medium (MA, Difco Laboratories, Detroit, USA), throughout the first week at 22ºC without any light access, and then for 13 hours in ultraviolet light (UV). After that, the isolates were incubated in the same conditions as in the first week of the culture [De Gruyter and Noordelos 1992].

Culture medium and growth conditions. For the culture of *B. strasseri* the Mandels-Weber (1969) medium was used (g/dm^3) which contained: KH_2PO_4: 2.0; $MgSO_4 \times 7H_2O$: 0.5; $(NH_4)_2SO_4$: 1.4; yeast extract (Sigma, St. Louis, USA): 1.,0; $CaCl_3$: 0.3; Tween 80: 1.0; trace mineral solution (0.5 ml) containing: $FeSO_4 \times 7H_2O$: 5.0; $MnSO_4 \times H_2O$: 1.96; $ZnSO_4$: 1.66; distilled water 1000 ml. Citrus pectin E 440 (Pektowin Company, Poland) was used as the carbon source and it was added to the medium at a concentration of 10 g/dm^3. Cultures of three isolates of *B. strasseri* were carried out in Erlenmeyer flasks of 500 ml containing 100 ml of Mandels-Weber medium [1969]. The flasks were stoppered and sterilized in an autoclave at 110°C for 30 minutes. pH value was adjusted to 5 before autoclaving. After cooling the medium 1 ml of a suspension containing 3×10^5 conidia/ml was added to each flask. It was obtained by flushing the surface of 14-day-old cultures of the individual isolates of

B. strasseri with sterile distilled water containing 0.01 ml of Tween 80. Cultures were incubated at 28°C with agitations at 220 rpm, until the carbon source was completely used up (6 days) [Wesołowska-Trojanowska and Targoński 2012].

Determination of the pectinolytic activity. The culture filtrates obtained from each isolate of B. strasser*i* was centrifuged at 7000 g for 10 min at 22°C (Sigma Laboratory Centrifuge 5430R) to remove cells and other debris. The supernatant were used as the crude enzyme sources. Pectinolytic activity of the supernatant resulting from the fungal growth was assayed by the method of Miller [1959] using citrus pectin as substrate. The reaction mixture consisted of 1 ml of 1% pectin in 1 ml of 0.1 M acetate buffer of pH 5 and 1 ml of supernatant (crude enzyme solution). Control experimental tubes contained the same amount of substrate and 1 ml of supernatant. Both the experimental and control tubes were incubated at 40°C for 1 h. The reaction was stopped with 1.5 ml 3,5-dinitrosalycylic acid solution [Miller 1959] after which the mixture was boiled for 5 min and cooled. The colour was read at 550 nm using a spectrophotometer (Spekol 11). The amount of reducing sugar released was quantified using galactouronic acid as standard. The specific enzymes activity was calculated as the amount of enzymes required to release one micromole (1 µmol) equivalent of galactouronic acid per minute under the specific conditions of reaction.

Effect of pH on pectinases activity. To estimate the pH optimum, the activity was determined by carrying out the above standard assay at several pH values 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7 and 7.5. Acetate buffer (0.1 M) was used for determination of optimal pH range of pectinases. Three replicates were performed for each pH value.

Effect of temperature on pectinases activity and heat stability. The effect of temperature on pectinases activity was analyzed in acetate buffer (0.1 M) incubated at the following temperatures: 30, 40, 50, 60 and 70°C, at the optimum pH. The thermal stability was investigated by measuring the residual activity of pectinases after 40, 50, 60, 70 and 80 min of incubation at 40, 50, 60 and 70°C. Three replicates were performed for each temperature value.

RESULTS AND DISCUSSION

Isolates of *B. strasseri* on liquid medium Mandels-Weber formed light-brown, round or oblong pellets (phot. 1). Pectinolytic activity of the supernatant resulting from the fungal growth was assayed. The filtrates were characterized by different enzymatic activity, but the highest pectinolytic activity was shown by the isolate M 743 (fig. 1). These results are consistent with the results obtained in pathogenicity tests, where from all of the tested isolates, the highest values of infection index in all inoculation methods for mint stems and rhizomes were for isolate 743 [Zimowska 2012]. The high level of pathogenicity of the isolate, which determines faster possession of the host tissues, is undoubtedly linked to its ability to produce extracellular enzymes. Pectinolytic activity of filtrates of *B. strasseri* as well as proliferation of the mycelium confirms the ability of isolates to use pectin as the carbon source and secrete pectinases to the substrate. Similarly, the hydrolysis of pectin, as a carbon source was reported previously for *Phoma sorghina* [Akiyosoye and Oboh 2004], *Colletotrichum lindemuthianum* [Berthe et al.

1981], *Fusarium moniliforme* [Caprari et al. 1993], *F. oxysporum* f sp. *cicerri* [Perez-Artez and Verma 1989], and *Aspergillus niger* [Arotupin et al. 2012].

Phot. 1. Six-days-old pellets of *B. strasseri* M 743 cultivated on Mendels-Weber medium (Photo B. Zimowska)

Pectinolytic activity of enzymes increased with increasing pH until the optimum value of 4.7 was reached (fig. 2). The enzymatic activity decreased at lower or higher pH (fig. 2). The optimum pH range for the effect of most pectinases is within the range 4–6. Therefore, these enzymes function well in acidic environment. Results of this study are consistent with previous reports in the literature, saying that the optimum pH for the activity of pectinases produced by *Aspergillus niger* and *Rhizopus stolonifer* was respectively 4.8 and 5 [Gummadi and Panda 2003], while in the case *of Aspergillus foetidus* and *A. aculeatus* the optimum pH was 5 [Nayebyazdi and Ghanbary 2012].

The study showed that pectinases produced by *B. strasseri* were active over a temperature range from 30°C to 70°C, however, the highest activity was observed at 40°C (fig. 3). This result indicates that the optimum temperature for *B. strasseri* pectinases of activity is 40°C and it is the highest temperature at which the denaturing effect is not revealed yet. For *Penicillium frequentans* and *Rhizoctonia solani* the thermal optimum for the activity of pectolytic enzymes was similar and amounted 50°C [Fátima et al.

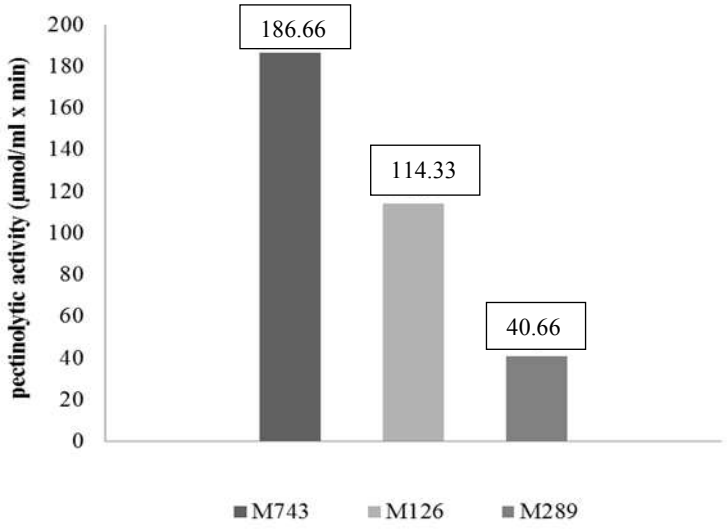

Fig. 1 Pectinases production by isolates of *B. strasseri*

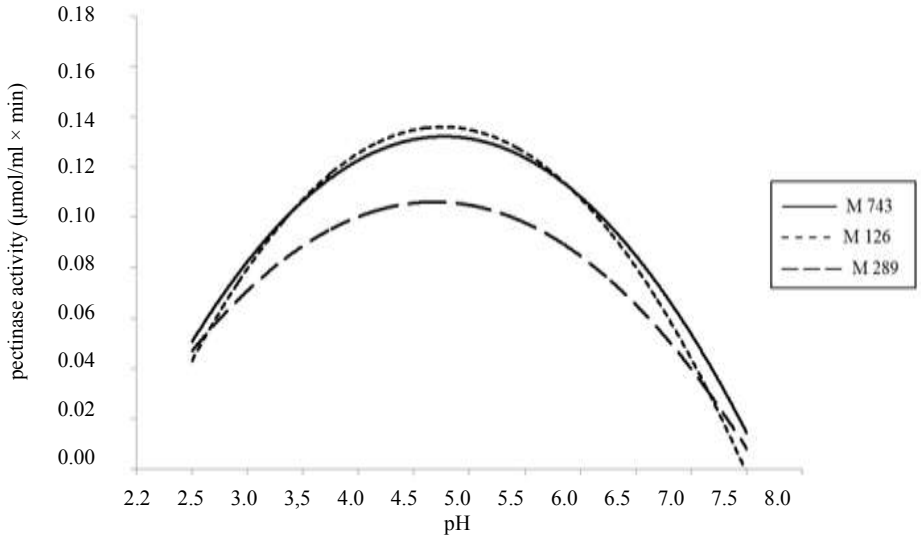

Fig. 2. Effect of pH on the activity of pectinases produced by *B. strasseri*

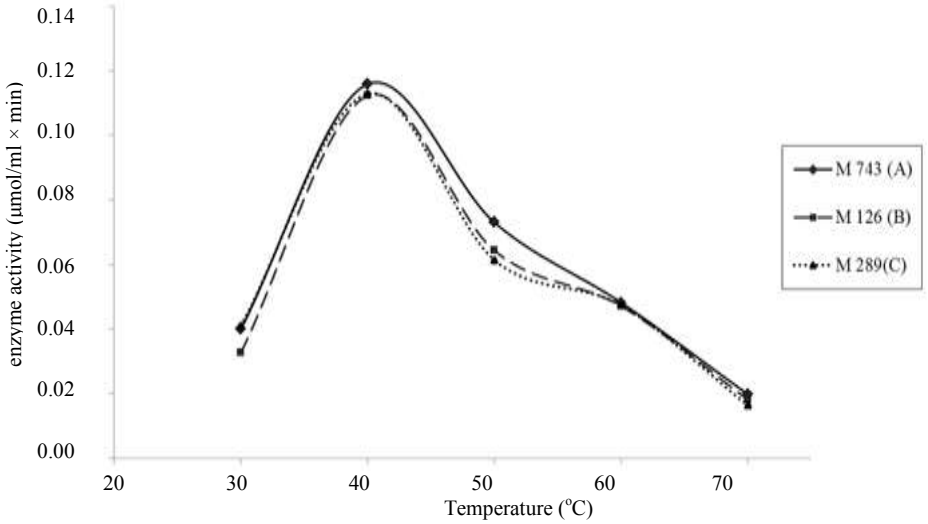

Fig. 3. Effect of temperature on the activity of pectinases produced by *B. strasseri*

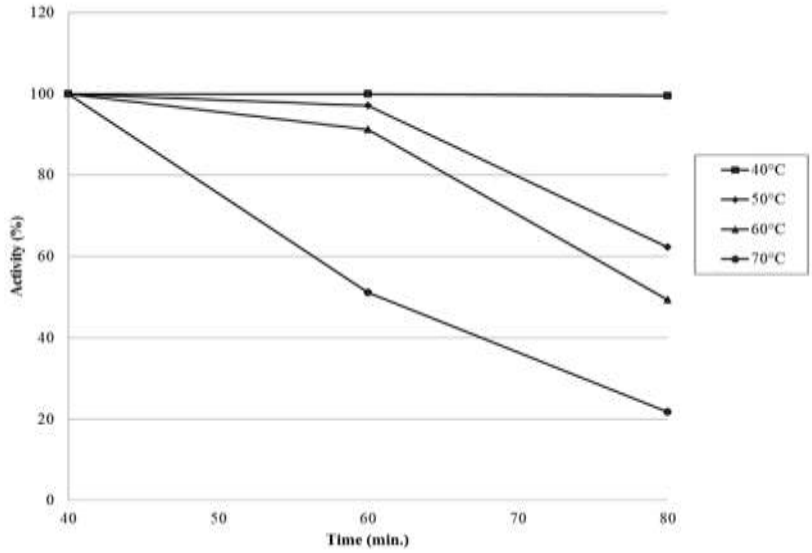

Fig. 4. Thermostability of the pectinases produced by isolate M 126 *B. strasseri* at temperatures ranging from 40 to 70°C

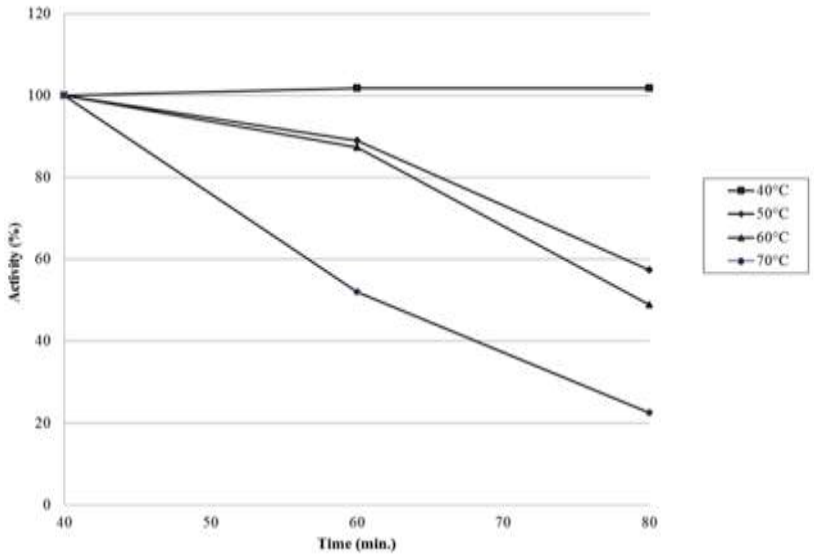

Fig. 5. Thermostability of the pectinases produced by isolate M 289 *B. strasseri* at temperatures ranging from 40 to 70°C

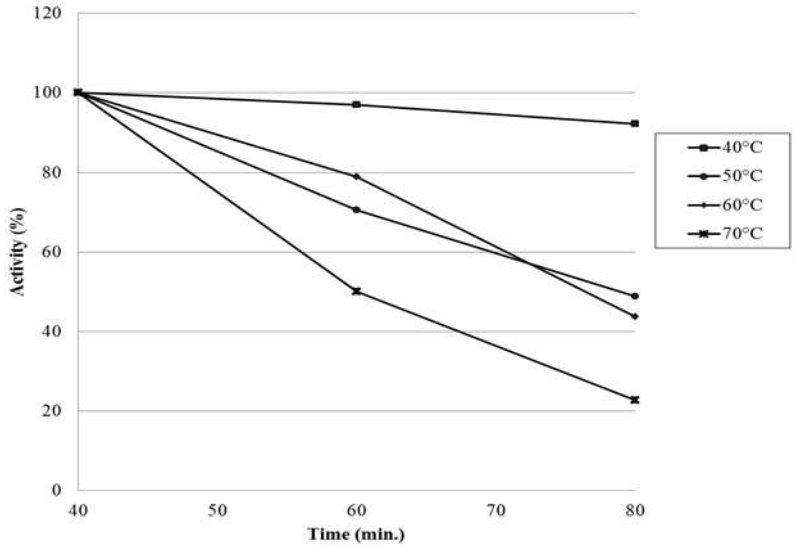

Fig. 6. Thermostability of the pectinases produced by isolate M 743 *B. strasseri* at temperatures ranging from 40 to 70°C

1996], while for *A. niger, A. japonicus*, and *Chaetomium globosum* to below 50°C [Acuña-Argüelles et al. 1995, Reddy and Sreeramulu 2012], and the thermal optimum for the activity of polygalacturonase produced by *Colletotrichum lindemuthianum*, *Penicillium variabile*, *Ganoderma lucidum* and *Venturia inequalis* was 40°C and for *A. flavus* it was 35°C [Barthe et al. 1981, Yoon et al. 1994, Arotupin 2007]. Variation of optimum temperature for pectinases production by the above-mentioned fungal species suggests a wide range of temperatures tolerated by these enzymes. In addition, the natural substrate, from which fungi are isolated and the physiological activity of the fungus can affect these differences [Arotupin 1991].

The research indicated a stable character of the pectolytic enzymes of three isolates of *B. strasseri* at 40°C (figs. 4–6). For the other temperature values a decrease of occurred enzyme activity over time, with the greatest loss of activity of the enzymes observed while heating at a temperature of 70°C (figs. 4–6). Reduction of polygalacturonase activity heated at 70°C was also observed in *P. variabile, Botryodiplodia theobromae* and *A. niger* [Oyede 1998, Ajayi et al. 2003].

It is well known that facultative pathogens such as *B. strasseri* [Zimowska 2012], which penetrate tissues, interrupt the host plant cell walls and plasma membranes thanks to the secretion of extracellular enzymes, which consequently lead to a disturbed metabolism of the cells and their death. In the pathogenesis of peppermint disease caused by *B. strasseri* a very important role is played by pectolytic enzymes because they are responsible for the breakdown of the central plaque, which is a kind of "mortar" binding parenchyma cells stems and rhizomes of peppermint. As a result, the structure of the tissue relaxes and the soft rot symptom was observed [Šutić and Sinclair 1991, Zimowska 2012]. The study showed that the pectolytic enzymes produced by *B. strasseri* are most active in strongly acidic medium at a temperature of 40°C.

CONCLUSION

Although some reports on production and characteristics of β-glucosidase by *B. strasseri* have been published, no information about characteristics of pectinases is available. In this study, we demonstrated that *B. strasseri* produce a thermostable pectinases at 40°C and optimum pH and temperature for pectinases activity were 4.7 and 40°C, respectively.

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AKTYWNOŚĆ PEKTYNOLITYCZNA *Boeremia strasseri* SPRAWCY CZARNEJ ZGNILIZNY ŁODYG I ROZŁOGÓW MIĘTY PIEPRZOWEJ

Streszczenie. Przeprowadzone badania dotyczą aktywności pektynolitycznej trzech izolatów *Boeremia strasseri.* Do hodowli grzyba użyto płynnej pożywki Mendels-Weber zawierającej 1% pektyny cytrusowej, jako źródło węgla. Aktywność pektynolityczna enzymów wzrastała wraz ze wzrostem pH do momentu osiągnięcia wartości optymalnej 4,7. Enzymy pektynolityczne *B. strasseri* były aktywne w szerokim zakresie temperatury od 30 do 70°C, jednak najwyższą aktywność zaobserwowano w temperaturze 40°C. Pektynazy były całkowicie stabilne w temperaturze 40°C przez 80 min. Największy spadek aktywności obserwowano podczas ogrzewania enzymów w temperaturze 70°C.

Słowa kluczowe: *Boeremia strasseri,* aktywność pektynolityczna, produkcja pektynaz, termostabilność, grzyby

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