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EXPERIMENTAL ARTICLES

Pectinase Production by a Brazilian Thermophilic Fungus *Thermomucor indicae-seudaticae* N31 in Solid-State and Submerged Fermentation[1]

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Abstract—Thermophilic organisms produce thermostable enzymes, which have a number of applications, justifying the interest in the isolation of new thermophilic strains and study of their enzymes. Thirty-four thermophilic and thermotolerant fungal strains were isolated from soil, organic compost, and an industrial waste pile based on their ability to grow at 45°C and in a liquid medium containing pectin as the only carbon source. Among these fungi, 50% were identified at the genus level as *Thermomyces, Aspergillus, Monascus, Chaetomium, Neosartoria, Scopulariopsis*, and *Thermomucor*. All isolated strains produced pectinase during solid-state fermentation (SSF). The highest polygalacturonase (PG) activity was obtained in the culture medium of thermophilic strain N31 identified as *Thermomucor indicae-seudaticae*. Under SSF conditions on media containing a mixture of wheat bran and orange bagasse (1 : 1) at 70% of initial moisture, this fungus produced the maximum of 120 U/ml of exo-PG, while in submerged fermentation (SmF) it produced 13.6 U/ml. The crude PG from SmF was more thermostable than that from SSF and exhibited higher stability in acidic pH.

Key words: pectinase, thermophilic fungus, *Thermomucor*, fermentation, thermostable enzyme, polygalacturonase.

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The majority of works on pectinase production by fungi refers to mesophilic strains [1], and only few studies have been carried out with thermophilic fungi [2–4]. However, the interest in enzyme production by thermophilic microorganisms has recently increased, especially due to their biochemical and physicochemical properties and inherent stability [4, 5]. On the other hand, a number of thermophilic fungal genera have been described, such as *Canariomyces*, *Chaetomium*, *Coonemeria*, *Corynascus*, *Dactylomyces*, *Malbrenchea*, *Melanocarpus*, *Myceliophthora*, *Paelomyces*, *Thermoascus*, *Thermomyces*, *Rhizomucor*, *Thermomucor*, *Scytalidium*, *Sporotrichum*, *Acrophiolophora*, *Stibella*, *Taloromyces*, and *Thielavia* [6]. Therefore, the isolation of thermophilic microorganisms and the subsequent identification of their thermostable enzymes is an useful approach to the development of new biotechnology tools.

Fungi have been considered among the most important industrial microorganisms mainly due to their capacity for secretion of the enzymes which carry out the degradation of plant cell walls, such as cellulases, xylanases, ligninases, and pectinases, as well as their ability to grow on solid substrates and therefore applicability to solid-state fermentation (SSF) [7]. However, some fungi secret higher levels of lignocellulolytic enzymes in submerged fermentation (SmF) than in SSF [8], The effect of culture conditions on enzyme production has been attributed to the water activity and humidity of the media [9], to the catabolic repression, to the available substrate levels [10] and to the structure of cell membrane, which may change accordingly to the growth conditions [11]. Thus, the evaluation of fermentation systems to be used and the understanding of the way the microorganisms adapt to SSF and SmF conditions are essential to the improvement of the enzyme production [12].

The major problem concerning the fermentation processes is the dissipation of the heat generated from microbial growth, which can inhibit the microbial metabolism. In this context, the use of microorganisms that are able to grow at higher temperatures could facilitate this process [13].

In the present investigation we report the isolation and selection of thermophilic fungal strains capable of growing in the medium with pectin as the only carbon source, and the use of one selected isolate for pecti-

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nase production in SSF and SmF by using agro-industrial waste as carbon source. Additionally, a comparative study on the physicochemical properties of the enzymes produced in both fermentative systems was presented.

MATERIAL AND METHODS

Microorganisms: isolation, identification and maintenance. The thermophilic and thermotolerant fungal strains were isolated from soil, organic compost and an industrial waste pile. Samples (2 g) were pooled and homogenized in sterile medium containing the following (g/l): citrus pectin, 10; $(NH_4)_2SO_4$, 1.4; K_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; and 10 (ml/l) of trace elements solution (5 mg/l $FeSO_4 \cdot 7H_2O$; 1.6 mg/l $MnSO_4 \cdot H_2O$; 1.4 mg/l $ZnSO_4 \cdot 7H_2O$; 2.0 mg/l $CoCl_2$); pH 5.0. The inoculated medium was incubated at 45°C for 24 h and a loop of the homogenized culture was then streaked on agar plates (30 g/l) containing the same medium, and incubated at 45°C for 24 to 72 h. The organisms with morphologically different colonies were isolated by repeated streaking. Pure cultures were subcultured on slants of the same medium for identification and enzymatic studies.

In general, identification of filamentous thermophilic fungi was based on the morphological and biochemical characteristics [14]. However, the selected fungal strain N31 was identified by both conventional and molecular approaches. Morphological characterization was carried out by colony observation through a stereomicroscope (Leica MZ6, Wetzlar, Germany) and by the observation of squashed lumps stained with lactophenol and cotton blue under a light microscope (Leica DM LS, Wetzlar, Germany) [15]. Molecular identification was performed by 28S rRNA D1/D2 gene sequencing and phylogenetic analyses as described by Sette [16].

The stock cultures were maintained at 7°C on Potato Dextrose Agar medium (PDA) under water and mineral oil, at room temperature.

Effect of temperature on fungal growth. In order to investigate the performance of strains at various temperatures, mycelial hyphae from pure cultures were spotted on agar plates and incubated at 27, 35, 40, 45, 50 and 55°C. The diameters of the colonies were measured at 12 h intervals.

Enzyme production in solid-state (SSF) and in submerged (SmF) fermentations. The solid substrates wheat bran, sugarcane bagasse, and orange bagasse (compressed mixture of pulp, peel and seeds) were obtained and processed as described previously [13]. Five grams of mixtures of wheat bran, orange and sugarcane bagasse at variable proportions (wt/wt) were placed in 250-ml Erlenmeyer flasks, and sterilized at 120°C for 40 min. This substrate was inoculated with 10 ml of a spore suspension, consisting of spores obtained from 1-day agar slant cultures in a sterile nutrient solution y containing 10 g/1 $(NH_4)_2SO_4$ and 10 g/l $MgSO_4 \cdot 7H_2O$. The material was moistened to 70% with sterile distilled water. The fermentation was carried out at 45°C for 12 days. Every day, one flask was removed and the fermented material was mixed with 8 ml of distilled water per gram of fermented material, stirred for 30 min, filtered, and centrituged at 10000 *g*, at 6°C. The supernatant was used as a crude enzyme solution.

The submerged fermentation (SmF) was carried out in 125-ml Erlenmeyer flasks, each with 20 ml of medium containing the following (g/l): $(NH_4)_2SO_4$, 10; $MgSO_4 \cdot 7H_2O$, 10; $NH_4H_2PO_4$, 10; and supplemented with 20 g/l of the carbons source (citrus pectin with degree of esterification (D.E.) of 67% (kelco), wheat bran, orange bagasse, or a mixture (1 : 1) of wheat bran and orange bagasse). This substrate was inoculated with 10 ml of a spore suspension, consisting of spores obtained from 1-day agar slant cultures in a sterile nutrient solution of 10 g/l $(NH_4)_2SO_4$ and 10 g/l $MgSO_4 \cdot 7H_2O$. Fermentation was carried out on a rotary shaker at 100 rpm for 120 h at 45°C. Every 24 h, one flask was removed and the biomass was separated by filtering through Whatman No. 1 paper in a Biichner funnel. The filtrate was used for the evaluation of the enzyme activity.

The fermentation experiments were performed in triplicate and the results are reported as means.

Enzyme activity measurements. Exo-polygalacturonase (exo-PG) activity was assayed by the DNS method [17] to quantify the number of reducing groups, expressed as galacturonic acid, released by enzymatic action in a controlled reaction, carried out in a solution containing 1% of citrus pectin (67% esterified with methyl groups (Sigma)) in 0.2 M sodium acetate buffer (pH 5.5) at 60°C for 10 min. One unit of enzyme activity (U) was defined as the amount of enzyme required to release one umol of galacturonic acid per minute under these assay conditions.

Enzyme characterization. Optimal activity of PG was assayed as a function of pH, using acetate (pH 3.0–5.0), citrate–phosphate (pH 5.0–7.0), Tris–HCl (pH 7.0–8.5) and glycine–NaOH (pH 8.5–10.5) as buffers at 60°C, and 1% of citrus pectin (Sigma) with D.E. of 67% as substrate. The effect of temperature on PG activity was determined by incubating the reaction mixture at temperatures ranging from 35 to 75°C, at optimum pH. Both assays were carried out according to the procedure described above.

The thermal stability was investigated by measuring the enzyme activity after incubation of the enzyme solution for 1 h, in the absence of substrates, at temperatures between 30 and 70°C. Another experiment was carried out maintaining the crude enzyme solution at 60°C for 2 h, in the absence of substrates. Remaining PG activity was determined at optimum pH and temperature, using 67% D.E. citrus pectin as substrate.

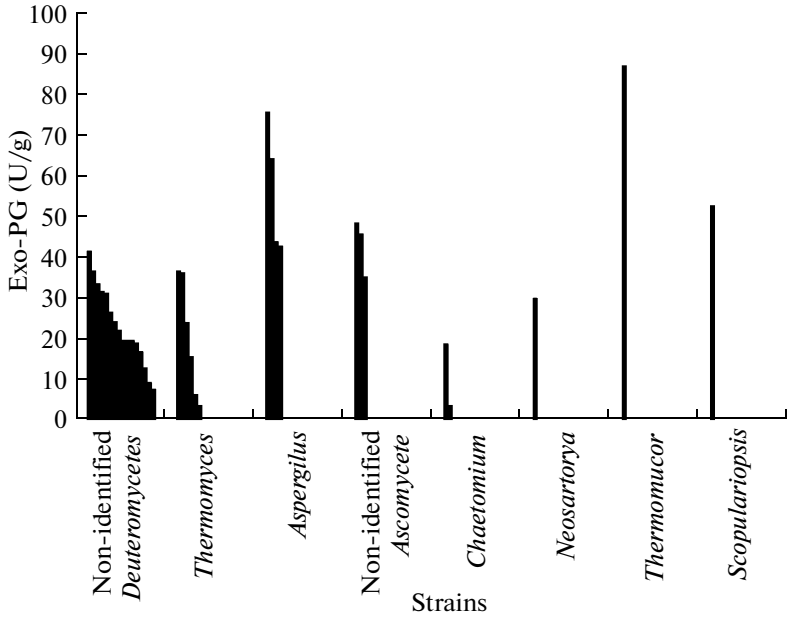

Fig. 1. Production of PG by thermophilic fungal strains isolated in SSF. Strains: non-identified deuteromycetes (16); *Thermomyces* (6); *Aspergillus* (4); non-identified ascomycete (3); *Chaetomium* (2); *Neosartoria* (1); *Scopulariopsis* (1); and *Thermomucor* N31 (01).

pH stability of the crude enzyme was evaluated by mixing the enzyme solution and 0.1 M buffers of pH 3.0–5.0 (sodium acetate), pH 5.0–7.0 (citrate–phosphate), pH 7.0–8.5 (Tris–HCl) and pH 8.5–11.0 (glycine–NaOH), to the final proportion of (1 : 1 vol/vol). These solutions were incubated at 25°C for 24 h. An aliquot was taken to determine the remaining enzyme activity at the optimum pH and temperature.

RESULTS AND DISCUSSION

Isolation, selection and identification of thermophilic pectinolytic fungal strains. Thirty-four thermophilic fungi with ability to grow at 45°C on a liquid medium containing pectin as the only carbon source were isolated from 40 collected samples. Some strains were identified as representatives of the genera *Thermomyces, Aspergillus, Chaetomium, Neosartoria, Scopulariopsis, and Thermomucor*, which are all described as thermophilic or thermotolerant. The number of thermophilic strains isolated was low considering the total microbial population that can be found in a decaying plant. However, according to Maheshwari [6], few thermophilic fungal species are known (around 30), in comparison with more than 75000 mesophilic forms already described.

When the capacity for pectinase production by the thermophilic fungi in SSF was evaluated, it was observed from 1 up to 87 U per gram of substrate. The highest PG activity (87.3 U/g) was achieved by *Thermomucor* N31 (Fig. 1), which was selected for use in other assays and for species identification procedures.

Data derived from BLAST analysis (28S rDNA D1/D2 region) showed that strain N31 shared a high sequence similarity (99%) with the type strain of *Thermomucor indicae-seudaticae* (NRRL 6429) and that these two fungal strains form a cluster in the phylogenetic tree (Fig. 2) supported by a 100% bootstrap value. In addition, data of the morphological identification were corroborative with the molecular data, allowing the affiliation of strain N31 to the genus and species *Thermomucor indicae-seudaticae* (*Fungi*; *Zygomycetes*; *Mucorales*; *Mucoraceae*).

The effect of incubation temperature on *T. indicae-seudaticae* N31 growth on solid medium is presented in Fig. 3. The maximum growth rate at 45–50°C confirms its thermophilic profile. According to Mouchacca's taxonomic review [18], fungi with optimal growth temperatures above the mesophilic range consist of a few *Rhizomucorales, Eurotiales, Sphaeriales*, and *Hyphomycetes*, and of agonomycete species. Among the *Rhizomucorales*, there are thermophilic species in the genera *Rhizomucor* and *Thermomucor*. In a recent revision of the genus *Absidia* (*Mucorales*, *Zygomycetes*), the fungus *T. indicae-seudaticae* formed a clade, based on the actin gene, with other thermotolerant or thermophilic species like *Rhizopus oryzae* and *R. microsporus* [19]. It is worth mentioning that, in the present study, results from rDNA phylogeny based on 28S rDNA D1/D2 of 21 *Mucorales* species were corroborative with Hoffmann's data based on the actin gene [19]. A 28S rDNA phylogenetic tree is represented in Fig. 2, in which *Mucorales* form a monophyletic clade to the *Mortierellales* outgroup, supported by a bootstrap of 100%, and the mesophilic

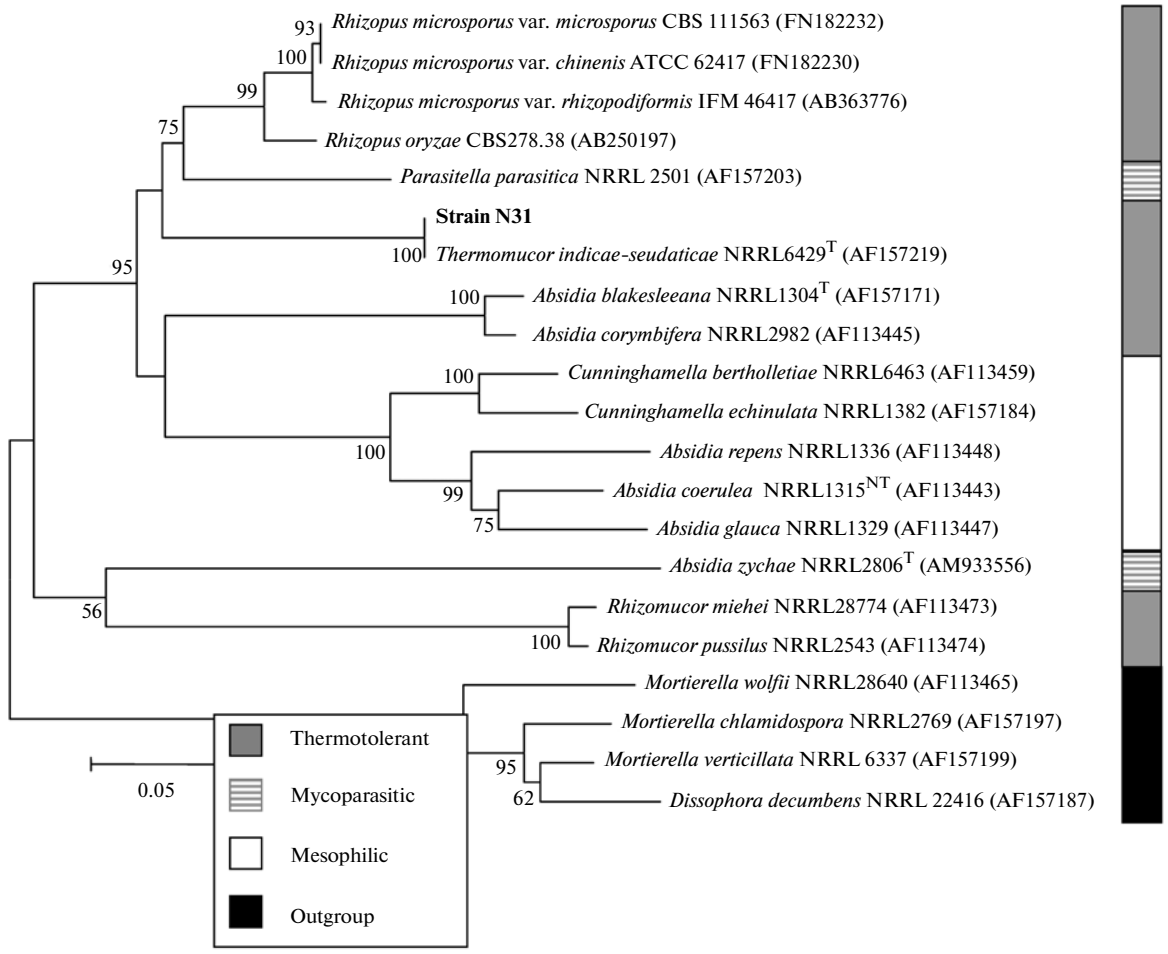

Fig. 2. Phylogenetic tree based on 28S rDNA D1/D2 analyses showing closest relatives of thermophilic fungal strain N31 (Kimura two-parameter model; neighbor-joining algorithm). Bootstrap values (1.000 replicate runs) greater than 50% are listed.

species are well-separated from the thermotolerant ones. Therefore, phylogenetic relationships based on actin [19] and 28S ribosomal DNA nucleotide sequences confirm the thermophilic profile of T. indicae-seudaticae N31.

Polygalacturonase production by *T. indicae-seudaticae* N31 in SSF and in SmF. After screening for pectinolytic activity, the strain *T. indicae-seudaticae* N31 was selected for subsequent SSF and SmF assays at 45°C. The table shows that the fungus exhibited exo-PG activity when grown on various agro-industrial wastes such as orange bagasse, wheat bran and sugarcane bagasse with 70% of initial moisture. The data showed that wheat bran was an effective medium for PG production. The addition of orange bagasse to wheat bran increased the enzyme activity up to 108 U/ml (mixture of wheat bran, orange bagasse, and sugar cane bagasse (2 : 2 : 1). Lignocellulosic material was successfully used as carbon source for SSF due to its degradability and to the presence of various nutrients besides the carbon source [20].

According to Martins [13] and Silva [21] the sugar cane bagasse increased production of pectinases by *Thermoascus aurantiacus* CBMAI 756 and *Penicillium viridicatum* RFC3 by enlarging the interparticle space, which improves the aeration, the nutrient and enzyme diffusion, and the heat transfer rates in the fermentation media. However, improvement of enzyme production by sugarcane bagasse addition was not observed in this study.

The total enzyme activity in the crude enzyme extracts obtained from SSF was higher than in SmF. There was no difference between the enzyme activities in SmF when media with wheat bran and a mixture of wheat bran and orange bagasse were used, but both conditions produced higher levels than that obtained with the medium containing citrus pectin (table).

Several investigators have observed lower production of extracellular enzymes in SmF than in SSF since the conditions of SSF are more similar to fungal growth conditions in nature [22]. The data obtained by Silva [23] showed that PG production by *P. viridicatum* RFC3 was lower in SmF than in SSF. A similar

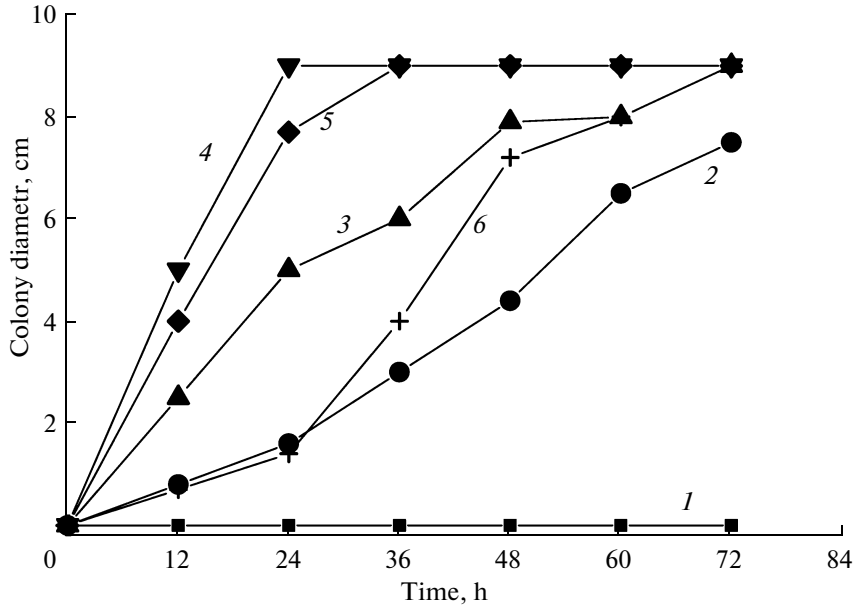

Fig. 3. Effect of temperature on mycelial growth of *T. indicae-seudaticae* N31 on solid PDA medium. *1*, 27°C; *2* , 35°C; *3*, 40°C; *4*, 45°C; *5*, 50°C; *6*, 55°C.

result was reported by Morita et al. [24], in which the maximum production of polygalacturonase was observed at a_w of 0.99, whereas the synthesis of β-glucosidase was increased at a_w between 0.96 and 0.98. The production of extracellular enzymes such as amylases, glucoamylases, xylanases, tannases and invertases was higher in SSF when compared with SmF [9]. The differential expression of proteins in SSF and SmF has been attributed to the effect of low a_w and physical barriers to the hyphal extension on the enzyme expression [22, 25]. The presence of proteases in the fermentation medium has also been associated with a more pronounced decrease in the activity of the target enzyme during the fermentation process in liq-

Polygalacturonase production by *T. indicae-seudaticae* N31 from SSF and SmF with different carbon sources

SSF			
Culture media composition (w/w)	Maximum PG production (U of enzyme per gram of substrate used)	Maximum PG production (U of enzyme in 40 ml of crude enzyme solution)	Cultivation time (h)
Wheat bran	98.0	488	192
70% wheat bran, 30% orange bagasse	87.3	436	24
50% wheat bran, 50% orange bagasse	105.0	524	48
50% wheat bran, 50% sugarcane bagasse	100.0	500	48
50% orange bagasse, 50% sugarcane bagasse	40.0	200	288
40% orange bagasse, 40% wheat bran, 20% sugarcane bagasse	108.0	540	48
70% orange bagasse, 20% sugarcane bagasse, 10% wheat bran	57.2	284	48
SmF			
Carbon source of media	(U of enzyme per ml of medium)	(U of enzyme in 20 ml of fermented medium)	Cultivation time (h)
Wheat bran 2%	12.8	256	72
Wheat bran 1% + orange bagasse 1%	13.6	272	96
Citrus pectin 2%	9.5	190	72

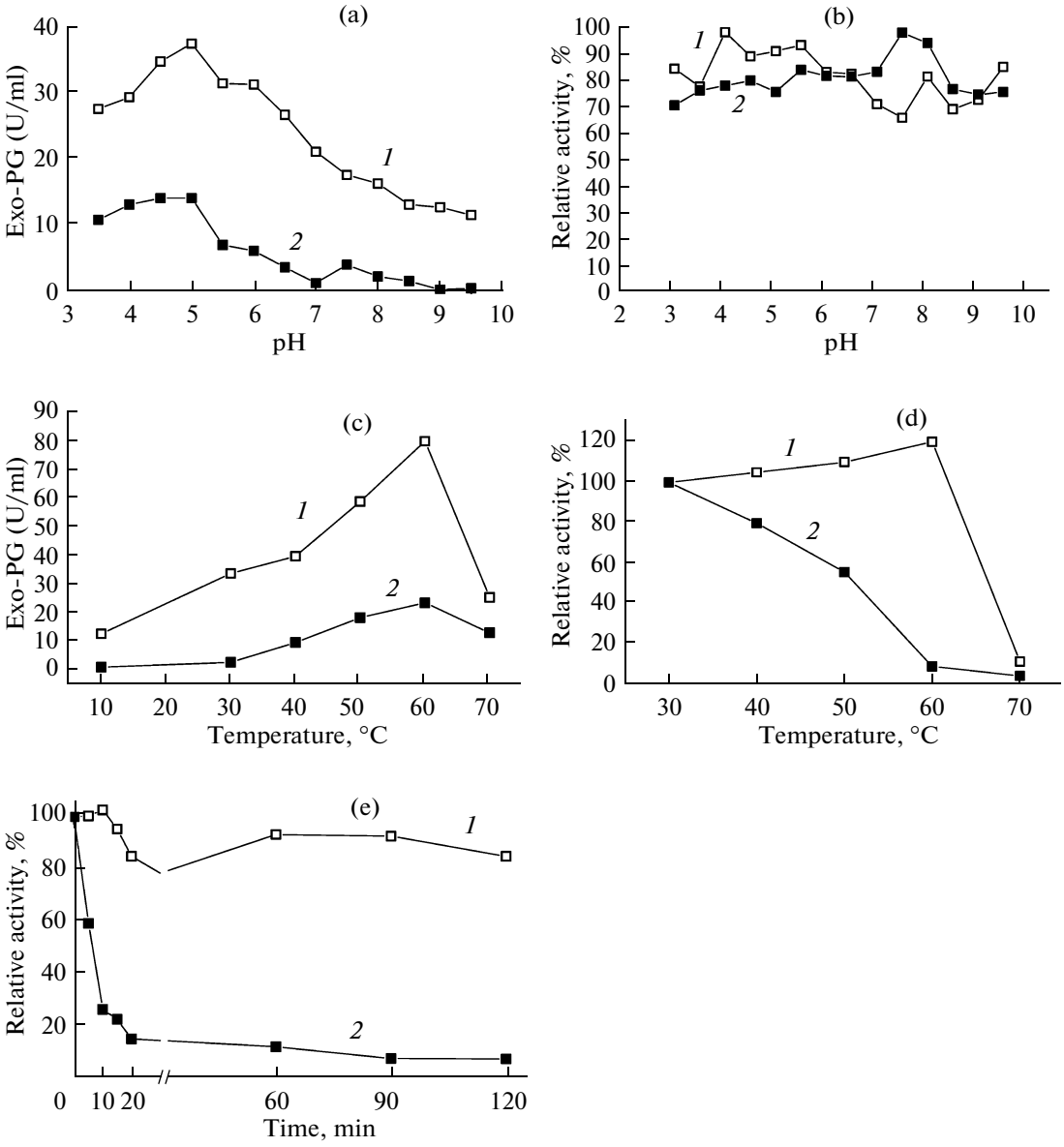

Fig. 4. Physicochemical properties of PG produced by *T. indicae-seudaticae* N31 in SSF and SmF. Effect of pH and temperature on the PG activity (a, c); effect of pH and temperature on the stability of enzymes in absence of substrate (b, d, e). *1*, submerged fermentation; *2*, solid state fermentation.

uid medium, reflecting a lower productivity in this type of culture [26].

In spite of this, the of PG activity observed in the present work was similar to the amount reported for PG production in SmF. *Aspergillus niger* produced 14.5 U/ml of pectinases in the medium containing citrus pectin [27]. The related *Aspergillus sojae* produced 15.5 U/ml of pectinases in the medium supplemented with maltrin [28]. On the other hand, it was lower than the value obtained by Patil and Dayanand [29] (30.3 U/ml) from *A. niger* DMF 27 when they used deseeded sunflower and glucose as carbon sources.

Properties of PG from SSF and SmF. Figure 4a shows that the optimum pH for PG from SmF condition was 5.0 while from SSF was 4.0–5.0. However, when in absence of a substrate, the PG from SmF was more stable in acidic pH while the PG from SSF was more stable in alkaline pH. Both enzymes retained above 80% of maximum activity in the pH range from 3.0 to 10.0 (Fig. 4b). The results reported by Acuña-Argüelles [30] showed that PG from *A. niger* obtained in SSF and SmF conditions were stable between pH 3.5 and 5.0 and 4.0 and 5.0, respectively.

The maximum PG activity was observed at 60°C (Fig. 4c) for both SSF and SmF and is consistent with

the optimum temperature reported for polygalacturonase from thermophilic *T. lanuginosus* [2] and *Sporotrichum thermophile* (55°C) [3].

In the absence of the substrate, the PG from SmF showed an increase of activity when incubated for 1 h at temperatures over 40°C, reaching 120% of the initial activity after 1 h at 60°C, while the enzyme from SSF lost 80% of its activity at 60°C (Fig. 4d). When the enzymes were incubated at 60°C for periods from 5 to 120 min, this difference was confirmed. The enzyme from SmF had the activity increased after 1 h at 60°C, although a slight decrease was observed during the first minute at this temperature. The PG from SSF lost 85% of activity after 20 min at 60°C (Fig. 4e). Data obtained by Acuña-Argüelles [30] revealed that the exo-PG produced by *A. niger* from SSF was more stable at 60°C than that obtained from SmF. The same was reported for amylase obtained from *Rhizopus homothalicus* [31]. However, the opposite was observed in this work, whereas PG from SmF showed to be more thermostable than that from SSF.

Intrinsic properties of microbial extracellular proteins such as temperature and pH optima for activity, thermostability, stability in different pH ranges and substrate affinity are often affected by the type of fermentation process used for their production [32]. Our data revealed that PG from SmF was more stable at acidic pH and more thermostable than that from SSF. It is worth mentioning that the main difference between the microorganism used in the present work and others reported in the literature is it thermophily. Probably, the fungus cultivation at 45°C could have influenced the enzymes properties.

Thus, the thermophilic fungus isolated from a compost pile and identified in the present study as *T. indicae-seudaticae* N31 produced higher quantity of PG in SSF than in SmF in accordance to the results reported for other fungal genera. However, it is important to highlight that the PG obtained in SmF was more stable at high temperature and acid pH than those obtained from SSF in contrast to several studies, in which enzymes from SSF displayed more stability at acid pH and high temperature than those from SmF.

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