Production of cold-active pectinases by three novel Cladosporium species isolated from Egypt and application of the most active enzyme

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Cladosporium parasphaerospermum, Cladosporium chlamydosporigenum, and Cladosporium compactisporum have all been discovered and characterized as new Cladosporium species. The three new species seemed to generate cold-active pectinases with high activity at pH 6.0 and 10 °C, pH 6.0 and 15 °C, and pH 5.0 and 15 °C, respectively, with the most active being C. parasphaerospermum pectinase. In submerged fermentation (SmF), C. parasphaerospermum produced the most cold-active pectinase with the highest activity and specific activity (28.84 U/mL and 3797 U/mg) after 8 days. C. parasphaerospermum cold-active pectinase was isolated using DEAE-Cellulose anion exchange resin and a Sephadex G 100 gel filtration column. The enzyme was purified 214.4-fold and 406.4-fold greater than the fermentation medium using DEAE-cellulose and Sephadex G 100, respectively. At pH 7.0 and 10 °C, pure pectinase had the highest activity (6684 U/mg), with $K_m$ and $V_{max}$ determined to be 26.625 mg/mL and 312.5 U/min, respectively. At 5 mM/mL, EDTA, MgCl$_2$, and SDS inhibited the activity of pure pectinase by 99.21, 96.03, and 94.45%, respectively. The addition of 10 U/mL pure pectinase enhanced the yield of apple, orange, apricot, and peach juice by 17, 20, 13, and 24%, respectively, and improved the clarity and colour of orange juice by 194 and 339%, respectively. We can now add cold-active pectinase production to the long list of Cladosporium species that have been identified. We also report three new species that can be used in biotechnological solutions as active microbial pectinase producers. Although further research is needed, these distinct species might be used to decompose difficult and resistant pectinacious wastes as well as clear fruit juices.

Cladosporium is one of the largest and most complex genera of hyphomycetes, which currently includes more than 728 names. Until recently, all types of unrelated dematiaceous hyphomycetes characterized by amero-to-phragmosporous conidia formed in acropetal chains had been referred to Cladosporium1. Species of Cladosporium are well adapted to spread easily in large numbers over long distances, therefore they are cosmopolitan and widely present in all various types of plants and other debris, mostly isolated from air, soil, seeds, grains, food, paint, textiles and other organic matter1–9. Several species of this genus are plant pathogenic causing leaf spots and other lesions10, or they occur as hyperparasites on some fungi11.

Cladosporium species are also known to be common endophytes12–14 as well as phylloplane fungi15–18. Some Cladosporium species including C. cladosporioides, C. chlorocephalum and C. uredinicola were recorded as entomopathogens of Aphids and whiteflies19,20. The Genus Cladosporium is considered to be a rich source of diverse and bioactive natural compounds21. Some species were reported to produce anticaner compounds such as L-asparaginase22, paclitaxel23 and useful enzymes including cellulases24 and pectinases25–27.

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Enzymes are nowadays in great demand in a variety of industrial applications, including food, detergent, paper, textiles, and organic chemical synthesis, due to their high efficiency and environmental friendliness. Furthermore, these enzymes are part of a well-established worldwide industry that is expected to grow to US$6.3 billion by few years. The current trend is to utilize cold-active enzymes to lower the temperature of industrial processes, allowing for energy savings and reduced carbon footprint, as well as the productive capacity that operate better at ambient or lower temperatures. Because they are (i) cost-effective, (ii) energy saving, (iii) capable of catalyzing processes without additional heat aid, and (iv) selectively inactivated by mild heat input.

In biotechnology, cold-active enzymes are used to prevent a range of unwanted reactions and restrict the loss of volatile components. As a result of these inevitable uses, the function of cold-active enzymes is expected to increase dramatically in the next years.

Pectin is one of the most numerous and complicated polysaccharides that make up the plant cell wall. It is a group of polysaccharides that contain at least seven structural components, the most well-known of which are homogalacturonan, xyloglacturonan, rhamnogalacturonan I, and rhamnogalacturonan II. Pectin is made up of a main chain of galacturonic acid residues bound by (14) links (homogalacturonan), or a mixture of galacturonic acid and rhamnose (rhamnogalacturonans) or galacturonic acid and xylose (xyloglacturonan). Various molecules, such as methyl, ethyl, and various sugar moieties (arabinose, rhamnose, galactose, and others), can then be replaced for the main chain. Pectin biodegradation necessitates the coordinated activity of multiple enzymes known collectively as pectinases, due to its complicated structure. Pectin methyl esterases, pectin acetyl esterases, polygalacturonases, polygalacturonate lyases, polygalacturonate lyases, rhamnogalacturonase, arabinase, and xyloglacturonases are all examples of pectinases.

Around 10% of the enzyme market is made up of pectinolytic enzymes, which are used in the juice, food, paper, and textile industries. Low temperatures (15°C) are employed in the juice industry to minimize cloudiness and bitterness in fruit juices in order to prevent the spread of harmful bacteria, preserve labile and volatile flavor components, and save energy. Researchers have been looking for pectinases that can act at low temperatures but also at low pH, because the pH of fruit juices and grape must be between 2.5 and 3.5. Pectinases are presently derived from mesophilic filamentous fungi, mostly Aspergillus species, however they work poorly below 35°C. Cold-active enzymes, on the other hand, have higher enzymatic activity than mesophilic enzymes at lower temperatures. As a result, the current research sought to develop cold-active pectinases from three newly-discovered Cladosporium species from Egypt, as well as purify, characterize, and use the most active pectinase in fruit juice production.

Results
Molecular studies. Cladosporium paraparaeochromum AUMC 10865. ITS: Based on a megablast search of NCBI GenBank nucleotide database, the closest hits using ITS sequence are Cladosporium clad-sporioide, Cladosporium parahalotolerans and Cladosporium halotolerans ([GenBank KJ76065, MK26909 and MK258720; identities = 554/556 (99.46%); Gaps = 1/556 (0%)]. ACT: the closest hits using ACT sequence are Cladosporium halotolerans and Cladosporium omanense ([GenBank MF084398 and MH176046; identities = 127/132 (96.21%); Gaps = 1/132 (0%)]. LSU: the closest matches using LSU are Digitaria exilis ([GenBank LR792838; identities = 1142/1152 (99.13%); Gaps = 10/1152 (0%) and Cladosporium delictatum ([GenBank J9732984 and J9732983; identities = 1140/1152 (98.96%); Gaps = 10/1152 (0%)]. Cladosporium chlamydosporigenum AUMC 11340. ITS: Based on a megablast search of NCBI GenBank nucleotide database, the closest hits using ITS sequence are Cladosporium subcinereum ([GenBank OK510626; identities = 551/554 (99.46%); Gaps = 2/554 (0%)] and Cladosporium floccosum ([GenBank MK460809; identities = 549/553 (99.28%); Gaps = 3/553 (0%)]. ACT: the closest hits using ACT sequence are Davidiella tassiana ([GenBank EU730605; identities = 229/240 (99.57%); Gaps = 0/240 (0%) and Cladosporium herbarum ([GenBank EF679510 and EF679511; identities = 228/230 (99.13%) and 227/230 (98.70%); Gaps = 0/230 (0%]). While compared to the aforementioned, the closest hits are Cladosporium intransovum ([GenBank EF679592; identities = 226/230 (98.26%); Gaps = 0/230 (0%)], Cladosporium herbarum ([GenBank EF679516; identities = 225/230 (97.83%); Gaps = 0/230 (0%) and Cladosporium versiforme ([GenBank KT600613; identities = 216/227 (95.15%); Gaps = 3/227 (1%)]. LSU: the closest hits using LSU sequences are Cladosporium herbarum and Cladosporium allicinum ([GenBank MH047193 and GU214408; identities = 1184/1199 (98.75%); Gaps = 13/1199 (1%)].

Cladosporium compactisporum AUMC 11366. ITS: Based on a megablast search of NCBI GenBank nucleotide database, the closest hits using ITS sequence are Cladosporium clad-sporioide, Cladosporium parahalotolerans and Cladosporium halotolerans ([GenBank ON045558 and MT367253; identities = 547/552 (99.09%) and 548/554 (98.92%); Gaps = 0/552 (0%) and 1/554 (0%)]. ACT: the closest hits using ACT sequence are Cladosporium clad-sporioide ([GenBank KY86457; identities = 232/232 (100%); Gaps = 0/232 (0%], Cladosporium proteacearum (ex-type) ([GenBank MZ344213; identities = 223/229 (97.38%); Gaps = 0/229 (0%], Cladosporium deviae (ex-type) ([GenBank MZ344212; identities = 212/216 (98.15%); Gaps = 1/216 (0%], and Cladosporium clad-sporioide (ex-type) ([GenBank HM148490; identities = 220/230 (95.65%); Gaps = 0/230 (0%]. LSU: the closest hits using LSU sequence are Cladosporium deli-catum and Cladosporium uredinicola ([GenBank J732985 and EU019264; identities = 1206/1221 (98.77%); Gaps = 12/1221 (0%], Toxicocladosporium irritans (ex-type) ([GenBank EU040243; identities = 1184/1221 (96.97%); Gaps = 12/1221 (0%], and Rachicladosporium inconspicuum (type) ([NG_059443; identities = 1159/1224 (94.69%); Gaps = 16/1224 (1%)].

Phylogenetic analyses. Descriptive statistical parameters of phylogenetic analyses and calculated tree scores for each analyzed sequence locus are summarized in Table 1. The constructed phylogenetic trees for ITS, ACT and LSU are shown in Figs. 1, 2, 3, respectively.
Taxonomy

Cladosporium parasphaerospermum sp. nov. Moharram AM, Zohri AA, Hesham A, Maher MA and Al-Bedak OA.

MycoBank. MB 844532.

Etymology. Name refers to globose to subglobose conidia near to that of C. sphaerospermum.

Holotype. Egypt, Beni Suef, Air, Maher MA, AUMC 10865. Ex-type culture: EMCCN: 2062.

Macroscopic and microscopic characteristics. Colonies on PDA reaching 18–20 mm diameter after 7 days at 28 °C, raised at the center, radially sulcate, radially furrowed under the colony, olive, olive green at the center (2F6). Margin curled, paler than the colony center (3F8). Sporulation profuse. Exudates absent. Colonies on SNA reaching 17–19 mm in diameter after 7 days at 28 °C, flat, slightly raised at the center, circular, olive, olive green (3F4-6). Margin entire. Sporulation abundant. Exudates absent. On OA colonies attaining 16–19 mm in diameter after 7 days at 28 °C, circular, flat, somewhat lanuginose, dark olive, dark olive green (1F4-6). Margin entire. Sporulation abundant. Exudates absent. Mycelium abundantly formed, branched, 3–5 µm wide, septate, pale brown to brown, smooth. Conidiophores macronematous, micronematous, abundantly formed, arising terminally or laterally, more or less straight to flexuous, cylindrical, pale brown to brown, smooth, septate, commonly (− 35) 75–100 × 3–5 µm (av. 87.5 × 4) µm (n = 50), not constricted at septa. Ramoconidia integrated, terminal, intercalary, cylindrical, smooth, thick-walled, 0–1 septate, 8–18 × 3–5 µm (av. 13 × 4) µm (n = 50), with 1–3 loci per cell. Loci usually confined to small lateral shoulders, protuberant, conspicuous, short cylindrical, 1–2 µm wide, up to 1–2 µm high. Conidia brown to dark brown, smooth, thick-walled, globose, subglobose, lemon-shaped, 0–septate, 4–6 × 3–5 µm (av. 5 × 4) µm (n = 50). Chlamydospores not formed (Fig. 4).

Cladosporium chlamydosporigenum sp. nov. Moharram AM, Zohri AA, Hesham A, Maher MA and Al-Bedak OA.

MycoBank. MB 844533.

Etymology. Refers to the formation of chlamydospores in culture.

Holotype. Egypt, Sohag, Grapevine fruits, Maher MA, AUMC 11340. Ex-type culture: EMCCN: 2332.

Macroscopic and microscopic characteristics. Colonies on PDA reaching 15–17 mm diameter after 7 days at 28 °C, raised at the center, wrinkled, irregular, olive, olive green (3F6). Margin undulate, narrow, paler than the center (3F6). Sporulation abundant. Exudates absent. Colonies on SNA reaching 9–11 mm in diameter after 7 days at 28 °C, flat, filamentous, olive, olive green (3E2). Margin filiform, narrow, (3E6-7). µm (av. 250 × 5) µm (n = 50), not constricted at septa. Ramonoconidia integrated, terminal, intercalary, cylindrical, smooth, thick-walled, 0–1 septate, 8–18 × 3–5 µm (av. 13 × 4) µm (n = 50), with 1–3 loci per cell. Loci usually confined to small lateral shoulders, protuberant, conspicuous, short cylindrical, 1–2 µm wide, up to 1–2 µm high. Conidia brown to dark brown, smooth, thick-walled, globose, subglobose, lemon-shaped, 0–septate, 4–6 × 3–5 µm (av. 5 × 4) µm (n = 50). Chlamydospores not formed (Fig. 5).

Cladosporium compactisporum sp. nov. Moharram AM, Zohri AA, Hesham A, Maher MA and Al-Bedak OA.

MycoBank. MB 844534.

| Parameter                                         | ITS          | ACT          | LSU          |
|---------------------------------------------------|--------------|--------------|--------------|
| Number of sequences included                      | 32           | 24           | 18           |
| Number of alignment positions                     | 566          | 226          | 1373         |
| Number of parsimony informative characters (PIC)  | 29           | 59           | 20           |
| Length of tree/number of steps                    | 135          | 188          | 83           |
| Consistency index (CI)                            | 0.733333     | 0.70518      | 0.777778     |
| Retention Index (RI)                              | 0.953488     | 0.899477     | 0.793103     |
| Rescaled consistency index (RC)                   | 0.699225     | 0.631074     | 0.616858     |
| Number of equally parsimonious trees retained     | 8            | 1            | 7            |
| Maximum log likelihood                            | −1310.26     | −946.35      | −2060.50     |

Table 1. Statistical parameters representing phylogenetic studies performed on three distinct loci’s sequence alignments.
Figure 1. Maximum likelihood phylogenetic tree generated from ML/MP combination analysis based on alignment of ITS sequences of *C. parasphaerospermum* AUMC 10865, *C. chlamydosporigenum* AUMC 11340 and *C. compactisporum* AUMC 11366 with the most similar sequences belonging to *Cladosporium* in GenBank database. Sequences of species in this study are in blue color. Bootstrap support values (1000 replications) for ML/MP combination equal to or greater than 50% are indicated at the respective nodes. The tree was rooted to sequence of *Cercospora beticola* CBS 116456 as outgroup (in red color).

**Etymology.** Refers to the compact conidial chains.

**Holotype.** Egypt, Qena, Air, Maher MA, AUMC 11366. Ex-type culture: EMCCN: 2358.

**Macroscopic and microscopic characteristics.** Colonies on PDA attaining 25–28 mm after 7 days at 28 °C, raised, umbonate, circular, olive to olive green (3E3-3F4). Margin entire, narrow, about 3.0 mm in width, paler than the colony center (3E1-3). Sporulation profuse. Exudates absent. Colonies on SNA attaining 17–20 mm diameter after 7 days at 28 °C, raised, umbonate, olive to olive green (3F6-7). Margin entire, about 3.0 mm in width, paler than the colony center (3E2-3). Sporulation abundant. Exudates absent. Colonies on OA attaining 19–23 mm in diameter after 7 days at 28 °C, raised, umbonate, lanuginose, olive grey (2E1-2). Margin undulate, narrow, dark olive grey (2F2). Sporulation abundant. Exudates lacking. Mycelium abundantly formed, branched, 3–5 µm wide, septate, swollen, pale brown to brown, smooth. Conidiophores macronematous and micronematous, abundantly formed, arising terminally or laterally, more or less straight to flexuous, nodulose, geniculate at the upper part, cylindrical, pale brown to brown, smooth, septate, branched, 100–300 µm × 3.0–6.0 µm (av. 200 × 4.5) µm (n = 50), smooth, 0–1 septa with 1–3 loci per cell. Loci usually confined to small lateral shoulders, protuberant, conspicuous, short cylindrical, 1 µm wide, up to 1–2 µm high. Conidia formed in compact and branched chains, pale brown, subglobose, obovoid to ellipsoid, smooth, 0–septate, 4–6 × 3–4 µm (av. 5 × 3.5) µm (n = 50). Chlamydospores not formed (Fig. 6).

**Optimization of cold-active pectinases production by the three *Cladosporium* strains.** Effect of pH and temperature on pectinase production. *Cladosporium paraxanthoxylum* AUMC 10865, *Cladosporium chlamydosporigenum* AUMC 11340, and *Cladosporium compactisporum* AUMC 11366 cold-active pectinase production was investigated in this work by altering the pH of the fermentation medium between pH 3.0 and 10.0 each at 5°, 10°, and 15°. *Cladosporium paraxanthoxylum* AUMC 10865 produced the most pectinase (26.3 ± 2.1 U/mL) at pH 6.0 and 10 °C (Fig. 7), whereas *C. chlamydosporigenum* AUMC 11340 produced the
most (24.63 ± 2.5 U/mL) at pH 6.0 and 15 °C (Fig. 8), and C. compactisporum AUMC 11366 (21.93 ± 2.3 U/mL) at pH 5.0 and 15 °C (Fig. 9).

Effect of nitrogen source and incubation time. Pectinase production by Cladosporium parasphaerospermum AUMC 10865 was enhanced (28.84 ± 2.7 U/mL) after 8 days incubation using sodium nitrate as a nitrogen source.
(Fig. 10). While, ammonium chloride was found to be best for pectinase production by *Cladosporium chlamydosporigenum* AUMC 11340 (26.6 ± 1.28 U/mL), and *Cladosporium compactisporum* AUMC 11366 (24.01 ± 1.76 U/mL) after 9 days (Figs. 11, 12).

**Production of cold-active pectinase by C. parasphaerospermum AUMC 10865 in SmF.** In submerged fermentation at the optimum conditions, the three fungi produced pectinases at a rather high output. *Cladosporium parasphaerospermum* generated 5.6 g of pectinase powder per liter of fermentation media, followed by C.
Figure 6. *Cladosporium compactisporum* (AUMC 11366). (A–C) 7-day-old colonies on PDA, SNA and OA at 25 °C. (D–F) Geniculate and swollen conidiophore bearing compact chains of conidia. Scale bar = 20 µm.

Figure 7. Effect of pH at 5, 10, and 15 °C on the pectinase production by *Cladosporium parasphaerospermum* AUMC 10865.

Figure 8. Effect of pH at 5, 10, and 15 °C on the pectinase production by *Cladosporium chlamydosporigenum* AUMC 11340.
Figure 9. Effect of pH at 5, 10, and 15 °C on the pectinase production by Cladosporium compactisporum AUMC 11366.

Figure 10. Effect of nitrogen source and incubation time on the pectinase production by C. parasphaerospermum AUMC 10865 at pH 6.0 and 10 °C after 8 days.

Figure 11. Effect of nitrogen source and incubation time on the pectinase production by C. chlamydosporigenum AUMC 11340 at pH 5.0 and 10 °C after 9 days.
Chlamydosporigenum at 3.65 g and C. compactisporum at 2.85 g (Fig. 13). For purification and use, the C. parasphaerospermum AUMC 10865 pectinase that was the most cold-active was chosen.

Purification of pectinase produced by Cladosporium parasphaerospermum AUMC 10865. The Cladosporium parasphaerospermum AUMC 10865 pectinase was homogenized using a number of techniques. Initial partial purification of the enzyme involved adding solid ammonium sulphate to the cell-free supernatant. The fraction with a salt saturation of 70% showed pectinase activity. This fraction was dialyzed with citrate buffer (pH 6.0)
and freeze-dried before being applied to the anion exchanger (DEAE-Cellulose), which was pre-equilibrated with 50 mM citrate buffer (pH 6.0). The proteins were extracted using a gradient of NaCl (0–1.5 M).

**Purification profile of pure pectinase.** Pectinase activity was discovered in fractions 60–150 of a DEAE-Cellulose column (Fig. 14), which were pooled, concentrated, and dialyzed against citrate buffer (pH 6.0). This cycle of purification increased pectinase purity by 214.4-fold, with a specific activity of 1005.55 U/mg protein (Table 2). The fractions with the highest pectinase activity were pooled, condensed with a freeze drier, and loaded onto a Sephadex G-100 column (Fig. 15). The pectinase activity-highest fractions were pooled, concentrated, and dialyzed against citrate buffer (pH 6.0). With a specific activity of 1906 ± 65 U/mg protein, this phase of purification resulted in a 406.4-fold improvement in pectinase purity (Table 2).

**Effect of pH and temperature on the pure pectinase activity.** The activity of pure pectinase was further tested in the presence of various physical and chemical factors. The purified pectinase has an optimal pH of 7.0. The purified pectinase had the highest activity (4553 ± 124 U/mg) at pH 7.0 and 5 °C, which increased to 6684 ± 173 U/mg at 10 °C (Fig. 16).

**Effect of some ions and inhibitors on the pure pectinase activity.** The purified enzyme was sensitive to all salts tested at a concentration of 5 mmol/mL. Pectinase activity was significantly reduced by 99.21, 96.03, and 94.45% using EDTA, MgCl₂, and SDS, respectively in the reaction (Table 3).

| Purification steps | Fraction volume (mL) | Protein (mg/mL) | Total protein (mg) | Activity (U/mL) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification factor (fold) |
|-------------------|---------------------|-----------------|-------------------|----------------|-------------------|------------------------|-----------|--------------------------|
| Fermentation medium | 1000                | 0.1464          | 146.4             | 0.687          | 687               | 4.692                  | 100       | 1                        |
| Ammonium sulfate  | 6.5                 | 6.46            | 42                | 17.31          | 112.5             | 2.68                   | 16.37     | 0.57                     |
| DEAE-Cellulose    | 15.0                | 0.018           | 0.27              | 18.1           | 271.5             | 1005.55                | 39.52     | 214.4                    |
| Sephadex G-100    | 10.0                | 0.003           | 0.03              | 5.718          | 57.18             | 1906                   | 8.32      | 406.4                    |

**Table 2.** Purification profile of pure pectinase produced by *C. parasphaerospermum AUMC 10865* at pH 6.0 and 10 °C in SmF.
The current findings revealed that Michaelis–Menten constant ($K_m$) and the maximum reaction velocity ($V_{max}$) values for the pure pectinase were calculated as 26.625 mg/mL and 312.5 U/min (Fig. 17).

Fruit juice production by the pure pectinase. When compared to the control, the enzyme treatment for all of the fruit pulps used resulted in a significant improvement in juice yield, clarity, and colour. It was determined how enzyme treatments affect the extraction of apple, orange, apricot, and peach juices. Enzyme addition increased juice recovery in all fruits. The enzyme treatment of apple, orange, apricot, and peach resulted in a significant increase in juice yield of 16.45, 16.43, 15.93, and 8.73%, respectively. The results also revealed a significant

| Ions and inhibitors | Specific activity (U/mg) | Residual activity (%) |
|---------------------|-------------------------|-----------------------|
| Control             | $6684 \pm 173^a$         | 100$^a$               |
| Na$^+$              | $2387 \pm 78^g$          | 35.7$^f$              |
| K$^+$               | $2758.66 \pm 69^g$       | 41.27$^f$             |
| Fe$^{2+}$           | $583.53 \pm 43^g$        | 8.73$^g$              |
| Ca$^{2+}$           | $848.6 \pm 66^g$         | 12.7$^b$              |
| Ca$^{2+}$           | $3380 \pm 116^g$         | 46.83$^e$             |
| Mg$^{2+}$           | $265.2 \pm 21^g$         | 3.97$^g$              |
| Zn$^{2+}$           | $2970.6 \pm 117^g$       | 44.44$^d$             |
| Ni$^{2+}$           | $2652.5 \pm 95^g$        | 39.7$^g$              |
| Mn$^{2+}$           | $354.33 \pm 123^b$       | 53.17$^b$             |
| EDTA                | $53.0 \pm 8^m$           | 0.79$^b$              |
| SDS                 | $371.33 \pm 23^b$        | 5.55$^b$              |

Table 3. Effect of some ions and inhibitors (5 mmol/mL) on pectinase activity produced by C. parasphaerospermum (mean ± SD, n = 3). The results are expressed as the activity in the tested inhibitory conditions compared to the pectinase activity in the control without inhibitors (in bold). At the 0.05 level of probability, means in a column with the same letters are not statistically different. Significant values are in bold.

Figure 17. Line-weaver-Burk equation used for $K_m$ and $V_{max}$ calculation.

Table 4. Yield, clarity, colour and pH of apple, orange, apricot, and peach fruit juices treated with pure pectinase produced by Cladosporium parasphaerospermum AUMC 10865. At the 0.05 level of probability, means in a column with the same letters are not statistically different. Significant values are in bold.

Kinetic constants of the pure pectinase. The current findings revealed that Michaelis–Menten constant ($K_m$) and the maximum reaction velocity ($V_{max}$) values for the pure pectinase were calculated as 26.625 mg/mL and 312.5 U/min (Fig. 17).

Fruit juice production by the pure pectinase. When compared to the control, the enzyme treatment for all of the fruit pulps used resulted in a significant improvement in juice yield, clarity, and colour. It was determined how enzyme treatments affect the extraction of apple, orange, apricot, and peach juices. Enzyme addition increased juice recovery in all fruits. The enzyme treatment of apple, orange, apricot, and peach resulted in a significant increase in juice yield of 16.45, 16.43, 15.93, and 8.73%, respectively. The results also revealed a significant
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and C. parasphaerospermum supported by ITS rDNA and partial actin gene analyses, with 57 and C. halotolerans can be recognized from C. chlamydosporigenum separated in the ACT tree each by a single long branch, and active pectinase in this study is groundbreaking. Due to minor changes in methodology, it is difficult to compare which has up to 4 loci packed at the tip.

Distinct branch. It produces smaller ramoconidia (7–22 µm) with 1–3 loci than (15–50 µm), C. cladosporioides alongside intercalary swellings. (11–22 µm), as well as the formation of chlamydospores and the absence of head-like swellings with additional intercalary swellings. C. compactisporum was discovered in ITS tree as part of a moderately supported clade alongside C. cladosporioides and C. tenuissimum, and in the ACT tree as part of the C. salinae clade on a lengthy distinct branch. It produces smaller ramoconidia (7–22 µm) with 1–3 loci than C. cladosporioides (15–50 µm), which has up to 4 loci packed at the tip. C. compactisporum differs from C. tenuissimum in that it has geniculate and nodulose conidiophores with compact conidial chains, whereas most C. tenuissimum conidiophores are neither geniculate nor nodulose. On Oat agar, C. tenuissimum has a longer conidiophore (up to 900 µm) than C. compactisporum. Conidiophores of 100–300 × 3–6 µm in length and ramoconidia of 7–22 µm in length distinguish C. compactisporum from C. salinae, which has weakly differentiated conidiophores (25–50 × 2.5–3.5 µm) and smaller ramoconidia (9.5–13.5 × 2.5–3.5 µm).

The current research aimed to isolate cold-active pectinases from three novel Cladosporium species that could function at low temperatures. The three strains produced a considerable amount of cold-active pectinases, which were active at temperatures as low as 5 and 10 °C. This is the first report of cold-active pectinase production from psychrotolerant Cladosporium species that we are aware of.

Pectinase has lengthy been used in commercial food processing to degrade pectin and aid in various processing steps such as liquefaction, clarification, and juice extraction. Pectinases are among the most widely used enzymes, accounting for 40% of all food enzymes. It has been demonstrated that certain Cladosporium species generate active pectinases, which could be active at temperatures as low as 5 and 10 °C. This is the first report of cold-active pectinase production from psychrotolerant Cladosporium species that we are aware of.

Pectinases released by microorganisms account for approximately 25% of global food enzyme sales. The vast majority of which is derived from filamentous fungi, specifically Aspergillus niger. It is uncommon for filamentous fungi to produce pectinase activity below 40 °C. This is true even for filamentous fungi that are psychrophilic or psychrotolerant. Sclerotinia borealis, a pathogenic fungus prevalent in extremely cold locations that does not grow over 20 °C, generates pectinases with optimal activity at 40 °C. Mucor flavus is another example of a psychrotolerant fungus that generates pectinases with optimal activity at 45 °C.

To the best of our knowledge, there is just one case of a filamentous fungus generating pectinases with optimal activity below 40 °C in the literature. Botrytis cinerea, a phytopathogenic fungus, generates pectinases with activity at 34 and 37 °C. In this investigation, Cladosporium parasaephaespermon produced high quantity of pectinase with the maximum activity at pH 7.0 and 10 °C. Thus, this is the first study to purify and exploit cold-active pectinase from Cladosporium species, which might be great candidates for cold-active enzyme synthesis. Industrial pectinases generated from fungi are a blend of pectinolytic enzymes and other components, contamination mitigation, and eradication of any residual enzyme activity, which cause deactivation of enzyme when temperature is raised, are driving this shift in trend.

Because of their biodegradability, non-toxicity, high selectivity, and high yields, microbial enzymes are superior to chemical synthesis. The global enzyme market was valued at $9.9 billion in 2019 and is expected to grow at a 7.1% annual rate from 2020 to 2027. The majority of commercial enzymes, including pectinase, are now mesophilic or thermophilic. In the food sector, and particularly in the fruit processing sector, there has been an increasing desire to replace high-temperature procedures with low-temperature processes. Specific economic and environmental benefits, such as energy savings, retention of biologically inert and aromatic fragrance components, and environmental benefits, are driving the shift towards cold-active enzymes.

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Pectinase activity in a pure preparation of Cladosporium parasaephaespermon was described and evaluated in this work for its potential application in fruit juice clearing. The use of Cladosporium parasaephaespermon pectinase improved juice recovery in all fruits used. The enzyme treatment of apple, orange, apricot, and peach resulted in a considerable increase in juice output. The results also demonstrated a considerable improvement in the purity and color of the juice obtained from orange fruit.
Conclusion
In the current study, three novel *Cladosporium* species were introduced and described as *Cladosporium parasphaerospermum*, *C. chlamydosporigenum*, and *C. compactisporum*. The three novel species appeared to produce cold-active pectinases that had high activity at pH 6.0 and 10 °C, pH 6.0 and 15 °C, and pH 5.0 and 15 °C, respectively, of which *C. parasphaerospermum* pectinase was the most active. The enzyme was purified by 214.4-fold and 406.4-fold by DEAE-Cellulose and Sephadex G 100, respectively. The highest activity of the pure pectinase was gained at pH 7.0 and 10 °C. *Km* and *Vmax* were calculated to be 26.625 mg/mL and 312.5 U/min, respectively.

The use of pure pectinase boosted the yield of apple, orange, apricot, and peach juice and improved the clarity and colour of orange juice. We can now add cold-active pectinase production to the long list of *Cladosporium* species that have been identified. We also report three new species that can be used in biotechnological solutions as active microbial pectinase producers. Although further research is needed, these distinct species might be used to decompose difficult and resistant pectinacious wastes as well as clear fruit juices.

Materials and methods
Isolation and maintenance of *Cladosporium* strains. Three *Cladosporium* isolates involved in the current study, of which two were isolated from air of Beni Suef and Qena cities and one from fruits of grapevine cultivated in Sohag city, Egypt. Settle plate method was employed for isolation of *Cladosporium* from air and direct plating technique for isolation from grapevine fruits. Czapek's Dox agar was used as an isolation medium. The isolation medium contained (g/L): Sucrose, 30; NaNO₃, 2; KH₂PO₄, 1; KCl, 0.5; MgSO₄.7H₂O, 0.5; FeSO₄. 0.01; ZnSO₄, 0.01; CuSO₄, 0.005; Rose Bengal, 0.05; chloramphenicol, 0.25; agar, 15 and the final pH 7.3. The newly discovered strains were preserved as frozen and lyophilized cultures and added to the culture collections of the Assiut University Mycological Centre (AUMC) and the Egyptian Microbial Culture Collections (EMCCN) as AUMC 10865 = EMCCN 2062 (Air, Beni Suef, Egypt), AUMC 11340 = EMCCN 2332 (Grapevine fruits, Sohag, Egypt), and AUMC 11366 = EMCCN 2358 (Air, Qena, Egypt). The new species were catalogued in the MycoBank with accession numbers MB844532, MB844533, and MB844534, respectively, along with their descriptions. Nexus file of the sequence alignments for all data sets were uploaded to Tree BASE http://purl.org/phylo/treebase/phylows/study/TB2:S30171?x-access-code=a18696d1afcf65925a63a31c3ebd045&format=html (Study no. 30171).

Morphological studies of the *Cladosporium* strains. For growth rate determination and phenetic description of colonies, strains were point inoculated on potato dextrose agar (PDA), synthetic nutrient agar (SNA) and oat meal agar (OA) and incubated at 25 °C for 14 days in darkness. Surface colours were rated using the colour charts.

Molecular identification of the *Cladosporium* strains. DNA extraction, PCR and sequencing of ITS, ACT and LSU. DNA isolation of *Cladosporium* isolates AUMC 10865, AUMC 11340 and AUMC 11366 was performed following CTAB method. The universal primers ITS1 and ITS4 were used for amplification of the internal transcribed spacer (ITS) region, ACT783R and ACT512F for amplification of ACT gene and LROR and LR781 for amplification of the large subunit (LSU). PCR was done following Al-Bedak and Moubasher.

Alignments and phylogenetic analyses. Sequences of *Cladosporium* species (ITS, ACT, LSU) in this study were compared to the type and ex-type species in GenBank. MAFFT (version 6.861b) with the default options was used for alignment of the three sequence sets (ITS, ACT, LSU) in this study. *Cercospora beticola* CBS 116456 was used as outgroup. Alignment gaps and parsimony uninformative characters were optimized by BMGE. Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using PhyML 3.0.

The robustness of the most parsimonious trees was evaluated by 1000 replications. The best optimal tree was edited using Microsoft Power Point (2016) and saved as TIF format.

Optimization of cold-active pectinase production by the *Cladosporium* strains. In a previous study, the three *Cladosporium* strains (AUMC 10865, AUMC 11340 and AUMC 11366) were found to be capable of producing cold-active pectinases in SmF at 10 °C. For maximization of pectinase production, pH, temperature, nitrogen source and fermentation time influencing pectinase production were optimized by varying parameters using two factors at a time (TFAT) for the three strains. The experiments were conducted in 250 mL Erlenmeyer flasks each with 50 mL fermentation medium (sucrose-free Czapek’s broth) supplemented with 1% citrus pectin as a sole carbon source. The flasks were inoculated separately with spore suspension (1%; v/v) obtained from 7-day-old of *Cladosporium* strains, and incubated under different operating conditions such as pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0), each at 5, 10, and 15 °C, and nitrogen source (peptone, yeast extract, sodium nitrate, ammonium sulfate, and ammonium chloride; each at 0.2%), at 1–10 days of incubation. Three replications of the experiment were performed.

Pectinase assay. The colorimetric approach was used to measure pectinase activity. Under static circumstances, 0.5 mL of adequately diluted cell-free supernatant was incubated with 0.5 mL of 1.0% citrus pectin (prepared in 50 mM Na-citrate buffer, pH 6.0) for 20 min at 10 °C. The mixture was boiled for 15 min after 2.0 mL of 3, 5-Dinitrosalicylic acid (DNS) was added. The colour created was evaluated at 540 nm for absorption. The
quantity of enzyme that catalyses the synthesis of 1 µmol of galacturonic acid per minute at the standard assay conditions was defined as one unit of pectinase.

Production of cold-active pectinase by Cladosporium parasphaerospermum in SmF. For pectinases production by Cladosporium parasphaerospermum AUMC 10865, the fungus was employed in Erlenmeyer flasks (500 mL) in SmF at the optimum conditions using the fermentation medium. Cladosporium species was inoculated with 1.5 x 10^6 spore/mL spore suspensions obtained from 7-day-old cultures. The incubation period lasted at 10 °C and 150 rpm.

Purification of the cold-active pectinase. Ammonium sulfate precipitation and dialysis. Following the incubation time, cell-free supernatant was recovered by centrifuging at 10,000 rpm for 10 min. At 4 °C, total protein was isolated using 70% saturated solution of ammonium sulphate. A freeze dryer (VirTis, model #6KBTES-55, NY, USA) was used to separate and lyophilize the precipitated protein. Lyophilized protein was dissolved in citrate buffer (pH 6.0) and dialyzed twice for 2 h at room temperature (cutoffs: 12–14 KD) against deionized water, eliminating the water each time, before being refrigerated overnight at 4 °C to remove salts and small molecules. The dialyzed protein was then lyophilized, and used in enzyme characterization experiments as partially purified fungal pectinase.

Ion exchange chromatography. A glass column (30 x 2.0 cm; 75 cm^3 bed volume) was filled with DEAE-Cellulose anion exchanger. After equilibrating the column with citrate buffer (50 mM, pH 6.0), a 6.0 mL sample was loaded onto it. With NaCl concentrations of 0, 0.1, 0.25, 0.5, 1.0, and 1.5 M, the enzyme was eluted with citrate buffer. The volume of the fractions was 5.0 mL. The pectinase activity was assessed using the previous approach. The fractions with the highest pectinase activity were mixed, concentrated, and kept for further study.

Gel filtration chromatography. In a glass column, Sephadex G 100 was packaged (55 x 2.5 cm; bed capacity 270 cm^3). The protein was eluted using citrate buffer (50 mM, pH 6.0) after this column was loaded with the concentrated sample (15 mL). Pectinase activity were evaluated using the techniques described previously in fractions of 5.0 mL volume. The pectinase-positive portions were mixed together, concentrated, and kept for future research.

Impact of pH, temperature and some ions and inhibitors on the pure pectinase activity. The impact of pH (3.0–10.0) at 5–15 °C on pure pectinase activity was investigated. The reaction mixture contained 100 µL pure enzyme and 900 µL pectin (dissolved in 50 mM buffer solution). After the reaction time (20 min), the reaction was terminated by introducing 2.0 mL of 3,5-dinitrosalicylic acid (DNS) 90, and the pectinase activity was determined for 10.0) at 5–15 °C on pure pectinase activity was investigated. The reaction mixture contained 100 µL pure enzyme and 900 µL pectin (dissolved in 50 mM buffer solution). After the reaction time (20 min), the reaction was terminated by introducing 2.0 mL of 3,5-dinitrosalicylic acid (DNS) 90, and the pectinase activity was determined for 10.0) at 5–15 °C on pure pectinase activity was investigated. The reaction mixture contained 100 µL pure enzyme and 900 µL pectin (dissolved in 50 mM buffer solution). After the reaction time (20 min), the reaction was terminated by introducing 2.0 mL of 3,5-dinitrosalicylic acid (DNS) 90, and the pectinase activity was determined for 10.0) at 5–15 °C on pure pectinase activity was investigated. The reaction mixture contained 100 µL pure enzyme and 900 µL pectin (dissolved in 50 mM buffer solution). After the reaction time (20 min), the reaction was terminated by introducing 2.0 mL of 3,5-dinitrosalicylic acid (DNS) 90, and the pectinase activity was determined.

Determination of kinetic constant (K_m and V_max). K_m (Michaelis–Menten constant) and V_max (maximum reaction velocity) values of the purified pectinase were determined by measuring enzyme activity at different concentrations of citrus pectin (1–16 mg/mL), using the Line-weaver-Burk equation.

Application of the pure pectinase in fruit juice production. Apple, orange, apricot, and peach pulps were employed for fruit production, clarity, colour, and pH using Cladosporium parasphaerospermum AUMC 10865’s pure pectinase. Each fruit pulp was treated with 10 U/mL pectinase enzyme (v/v), with untreated fruit pulps serving as controls. The processed fruit pulps were then incubated at 10 °C for 60 min. After inactivating the enzyme by boiling for 5 min, samples were recovered by centrifugation at 5000×g for 10 min. for clarity measurements. The juice yield was estimated by dividing the juice mass by the fruit mass.

Statistical analysis. Data were subjected to analysis of variance (ANOVA: two-factor with replication) followed by the Duncan’s multiple range test.

Data availability The newly discovered strains were preserved as frozen and lyophilized cultures and added to the culture collection of the Assiut University Mycological Centre (AUMC) and the Egyptian Microbial Culture Collection Network (EMCCN) as AUMC 10865 = EMCCN 2062 (Air, Beni Suef, Egypt), AUMC 11340 = EMCCN 2332 (Grapevine fruits, Sohag, Egypt), and AUMC 11366 = EMCCN 2358 (Air, Qena, Egypt). The new species were catalogued in the MycoBank with accession numbers MB844532 (MycoBank Typification; MBT 10007648), MB844533 (MycoBank Typification; MBT 10007649), and MB844534 (MycoBank Typification; MBT 10007649), respectively, along with their descriptions. Nexus file of the sequence alignments for all data sets were uploaded to TreeBASE http://purl.org/phylo/treebase/phylows/study/TB2:S30171x-access-code=a18696d1af659925a63a31c3ebd0458&format=html (Study no. 30171). The datasets generated and/or analyzed during the current study.
are available in the GenBank repository (https://www.ncbi.nlm.nih.gov/genbank) and MycoBank (https://www.mycobank.org/).

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A.M.M., A.-N.A.Z.: Supervision, revising; M.A.M.: Fungal isolation, enzymes production; A.E.H.: Editing, revising; O.A.A.-B.: Molecular work, data analysis, writing, revising; O.A.A.-B., M.A.M.: Enzyme purification and characterization; H.E.F.A.-R., M.A.M.: Enzyme application. All authors have read and agreed to the published version of the manuscript.

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