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Purification and properties of an endoglucanase from *Aspergillus niger* VTCC-F021

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Abstract: An extracellular endoglucanase (EG) from *Aspergillus niger* VTCC-F021 was purified 2.09-fold to homogeneity with a yield of 18.4%. The enzyme had a molecular mass of 31 kDa and a specific activity of 14.122 U/mg protein. Optimum temperature was observed at 55 °C and optimum pH at 5. The enzyme was stable up to 50 °C and from pH 5 to 6 with residual activity >80% and 60%, respectively. The kinetic constants K_m and V_{max} determined for EG, with carboxyl methyl cellulose as a substrate, were 8.5815 mg CMC/mL and 20.121 U/mg protein, respectively. EDTA increased EG activity by 35% at 5 mM and decreased activity by 12% at 15 mM. The metal ions Cu^{2+} and Fe^{2+} activated EG activity 112%–152% at 5–15 mM. EG showed a high resistance to Tween 20 and Tween 80 at 0.5%–2.0% (w/v) and to ethanol and methanol at 10%–20% (v/v) with a residual activity of >80%. The biochemical properties of EG demonstrated that this enzyme was quite different from those of *A. niger* strains. These results suggested that EG from *A. niger* VTCC-F021 could be used to investigate the efficacy of feed enzymes.

Key words: *Aspergillus niger* VTCC-F021, carboxymethyl cellulose, endoglucanase, purification, characterization

Introduction

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most common and renewable organic compounds in the biosphere. It has been used in the production of paper, clothes, food additives, and ruminant nutrients. Although cellulose is the most abundant carbohydrate on earth, relatively few animals are able to digest this polysaccharide efficiently (1) due to the function of cellulose-degrading enzymes, or cellulases. Since cellulases were identified, cellulose has emerged as a renewable energy source with enormous potential (2).

Cellulase is a complex enzyme system comprising 3 hydrolytic enzymes: endo-(1,4)-β-D-glucanase [endoglucanase (EG), endocellulase, CMCase (EC 3.2.1.4)], exo-(1,4)-β-D-glucanase [cellobiohydrolase, exocellulase, microcrystalline cellulase, avicelase (EC 3.2.1.91)], and β-glucosidase [cellobiase (EC 3.2.1.21)]. EG cleaves β-linkages at random, commonly in the amorphous parts of cellulose, while exoglucanase releases cellobiose from the nonreducing or reducing end, generally from the crystalline parts of cellulose, and glucosidase releases glucose from cellobiose and short-chain cellooligosaccharides (2). The complete hydrolysis of cellulose requires the synergistic action of these enzymes, which have different functions (3).

Cellulases have a broad variety of applications in the food, animal feed, brewing, paper pulp, detergent, textile, fuel, chemical, waste management,

and pollution treatment industries (2,4,5). Due to their broad industrial applications, EGs, 1 of 3 important cellulose-degrading enzymes, have been purified and characterized from a large number of microorganisms. However, only a few of these microorganisms produce significant quantities of extracellular enzymes capable of completely hydrolyzing crystalline cellulose in vitro (6). Fungi are the main cellulase-producing microorganisms. As one of the most potent cellulolytic enzyme producers, many filamentous *Aspergillus* strains have been investigated for EG production, purification, and characterization: *A. aculeatus* (7), *A. awamori* (8), *A. nidulans*, *A. niger* (9), *A. oryzae* (10), and *A. terreus* (11,12).

In a previous study we optimized the production of an EG from *A. niger* VTCC-F021 (13). Maximum production of the EG was achieved at 37 °C with an initial pH of 4.5 after growth for 120 h in a nutrient medium containing 0.4% (w/v) sugarcane bagasse as a carbon source, 1.4% (w/v) soybean powder as a nitrogen source, and 1% (w/v) carboxymethyl cellulose (CMC) as an inducer. The present work is focused on the purification and characterization of physicochemical and enzymatic properties of an EG from *A. niger* VTCC-F021 deposited at the Vietnam Type Culture Collection (http://imbt.vnu.edu.vn/vtcc/).

Materials and methods

Chemicals

CMC with an approximate molecular weight of 250 kDa and degree of substitution of approximately 7 carboxymethyl groups per 10 anhydroglucose units was obtained from Biochemika (Sigma Aldrich Co., St Louis, MO, USA). Sephadex G-100 was supplied by Phamarcia Co. (GE Healthcare, Uppsala, Sweden); Tween 20 and Tween 80 by Bio Basic Inc. (New York, USA); SDS by Sigma Aldrich Co. (St Louis, MO, USA), and Triton X-100 by Merck (Darmstadt, Germany). All chemicals were of analytical grade unless otherwise stated.

Strain cultivation

The *A. niger* VTCC-F021 strain from the Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam University Hanoi), was used for EG production. The strain was grown in 200 mL Erlenmeyer flasks containing 50 mL of nutrient medium with the following composition (g/L): K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; CMC, 10; sugarcane bagasse, 4; soybean powder, 14; and pH 4.5. The strain was grown at 37 °C for 5 days with 200 rpm rotary shaking, as previously described (13). The biomass was removed by centrifugation for 10 min at 6000 rpm, and the culture supernatant was used for EG purification.

Endoglucanase activity assay

EG activity towards CMC was measured by the appearance of reducing end groups in a CMC solution, as previously described (13). Glucose was used as the standard reduced sugar for concentration estimation. The amount of enzyme that released 1 µmol of glucose per minute under standard assay conditions was defined as 1 unit of EG activity.

Purification of endoglucanase

The cell culture was centrifuged for 10 min at 6000 rpm, and the protein from 15 mL of the culture supernatant was precipitated overnight with ammonium sulfate (70% saturation) under constant stirring. The resulting precipitate was collected by centrifugation at 12,000 rpm for 15 min and dissolved in 2.5 mL of 0.5 M acetate buffer. The supernatant was dialyzed against the buffer overnight with 3 changes. The dialyzed fraction (4 mL) was loaded onto a Sephadex G-100 column (2.6 × 6 cm) pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.5) at a flow rate of 25 mL/h and then washed with the same buffer. The protein was eluted with 20 mM potassium phosphate buffer (pH 7.5) containing a linear gradient of sodium chloride from 0–0.5 M at a flow rate of 25 mL/h; 2 mL fractions were collected. The fractions showing high glucanase activity were pooled and used as purified enzyme for characterization. All purification steps were carried out at 4 °C unless otherwise specified.

Gel electrophoresis and protein concentration

The homogeneity and molecular mass of the EG was determined by 12.5% SDS polyacrylamide gel electrophoresis (14) with Biometra equipment (Göttingen, Germany). Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Protein concentrations were measured by Bradford assay (15) using bovine serum albumin as a standard.

Kinetic parameters

The apparent kinetic parameters (V_{max} and K_m) were determined against 2–14 mg/mL of CMC as a substrate using Lineweaver-Burk plots.

Temperature and pH optimum

The pH and temperature optimum of the EG were determined by measuring the activity, as described above, using 100 mM sodium acetate buffer (pH 3–5.5) and 100 mM potassium phosphate buffer (pH 5.5–7) at 45 °C and in the temperature range 30–70 °C using 100 mM potassium phosphate buffer pH 5, respectively.

Temperature and pH stability

For the determination of temperature and pH stability the purified enzyme, 22 µg for each reaction, was preincubated at different temperatures from 30 to 60 °C at pH 5 and in a pH range (100 mM sodium acetate, pH 3–5; 100 mM potassium phosphate, pH 6–8) at 37 °C for 1–8 h, respectively. The residual activity was then determined.

Effect of metal ions, detergents, and organic solvents

Purified EG, 22 µg for each reaction, was preincubated in the presence of 5–15 mM of various metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}, and K^{+}) and EDTA, or in the presence of 10% – 30% (v/v) of different solvents (methanol, ethanol, isopropanol, n-butanol, acetone, and ethyl acetate), or in the presence of 0.5% – 2% (w/v) of different detergents (Tween 80, Tween 20, SDS, and Triton X–100) at 37 °C for 2 h. The residual activity was then determined.

Results and discussion

Purification of *A. niger* endoglucanase

The glucanase production of *A. niger* VTCC-F021 in the nutrient medium was 2.68 U/mL (specific activity, 5.52 U/mg protein) after growth for 120 h. This culture supernatant was precipitated, dialyzed, and subjected

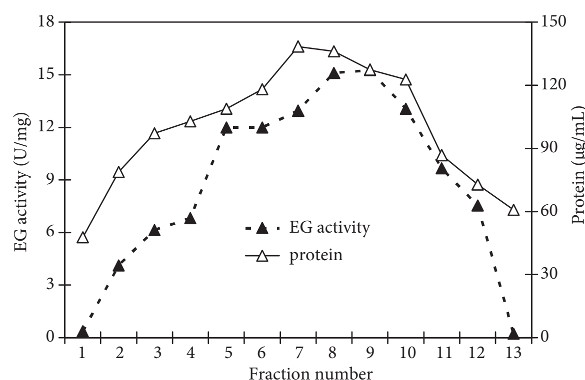

Figure 1. Sephadex G-100 gel filtration chromatography of EG from *A. niger* VTCC-F021.

to Sephadex G-100 gel filtration chromatography (Figure 1). The glucanase was purified with a factor of 2.09 and a yield of 18.4%. The purified enzyme gained specific activity of 14.122 U/mg (Table 1) and showed a single protein band on SDS-PAGE (Figure 2, lanes 3 and 4) as well as on native PAGE (data not shown), with a molecular mass of 31 kDa.

Other EGs from *Aspergillus* strains were purified to homogeneity using a similar purification scheme involving ammonium sulfate precipitation, acetone fractionation, and various types of chromatography (gel filtration: Sephadex G-100, ion exchange: DEAE-Sephadex A-50, affinity: Sepharose-4B, and exclusion). The EG from *A. niger* VTCC-F021 showed a different molecular mass (31 kDa) than EG from *A. niger* strains in other reports: 24 kDa (16), 26 kDa (17), 37 kDa (18), 40 kDa (19,20), 50 and 83 kDa (21), and 90.5 kDa (22). Furthermore, the molecular mass of EG from *A. niger* VTCC-F021 was also very different from the purified EG from other *Aspergillus* strains: *A. aculeatus* SM-L22, 24 and 39 kDa (7); *A. awamori* VTCC-F099, 32 kDa (8); *A. oryzae* VTCC-F045, 36 kDa (10); *A. terreus* M11, 25

Table 1. EG purification steps from crude enzyme of *A. niger* VTCC-F021.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude	5.955	40.215	6.753	100	1
$(NH_4)_2SO_4$	2.43	12.715	5.233	31.62	0.78
Sephadex G-100	0.524	7.4	14.122	18.4	2.09

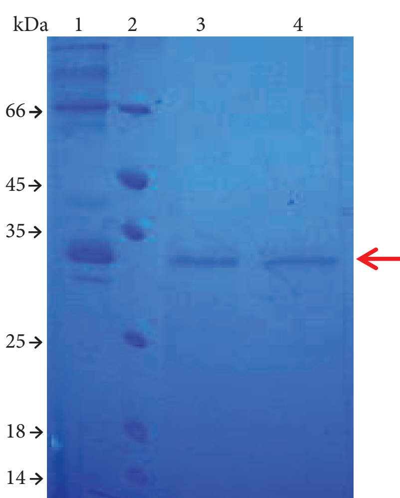

Figure 2. SDS-PAGE of the purified EG from *A. niger* VTCC-F021 through Sephadex G-100; lane 1: crude enzyme; lane 2: molecular mass marker; lane 3: fraction 8; lane 4: fraction 9.

kDa (11); and *A. terreus*, 78 and 80 kDa (23).

EG was purified 18-fold with a yield of 14% and a specific activity of 67 U/mg protein [*A. terreus* M11, (11)], 27-fold with a yield of 10.5% [*A. terreus* DSM 826, (12)], and 40-fold with a yield of 1.32% [*A. terreus* AN_1, (23)].

Kinetic parameters

The K_m and V_{max} values obtained for purified EG from *A. niger* VTCC-F021 were 8.5815 mg CMC/mL and 20.121 U/mg protein, respectively. The K_m value (8.5815 mg CMC/mL) was lower than that obtained for EGs from *A. niger* (52–80 mg/mL) (17) and *S. sclerotorium* (8.7 mg/mL) (24); however, it was higher than the values obtained for cellulases from *A. awamori* VTCC-F099 (5.83 mg/mL) (8) and *M. verrucaria* (0.5 mg/mL) (25). The V_{max} (20.121 U/mg protein) obtained for EG from *A. niger* VTCC-F021 was lower than that obtained for EGs from *A. awamori* VTCC-F099 (333.33 U/mg) (8) and *A. terreus* AN_1 (200 U/mg protein) (23) but higher than that obtained from *A. terreus* DSM 826 (4.35 U/mg protein) (12) when CMC was used as substrate.

Temperature and pH optimum

A. niger EG activity increased gradually from 1.549 U/mg (23%) at 30 °C to a maximum of 6.801 U/mg (100%) at 55 °C (Figure 3A) and then decreased gradually to 2.264 U/mg (33%) at 70 °C. The EG activity gradually increased from 3.569 U/mg (30%) at pH 3 to a maximum of 12.079 U/mg (100%) at pH 5 and then decreased gradually to 3.694 U/mg (31%) at pH 7 (Figure 3B).

Most EG from *Aspergillus* strains showed similar optimum temperature (55–70 °C) and pH (3.5–5.0) profiles: 50 °C, pH 4.8–5 for EG from *A. terreus* DSM 826 (12) and *A. awamori* VTCC-F099 (8); 55 °C, pH 5.5 [*A. oryzae* VTCC F045 (10)]; 55–70 °C, pH 3.5–4.0 [*A. aculeatus* SM-L22 (7)]; 60 °C, pH 2 [*A. terreus* M11 (11)]; 60 °C, pH 4 [*A. terreus* AN_1 (23)]; and 70 °C, pH 6–7 [*A. niger* IFO31125 (19)].

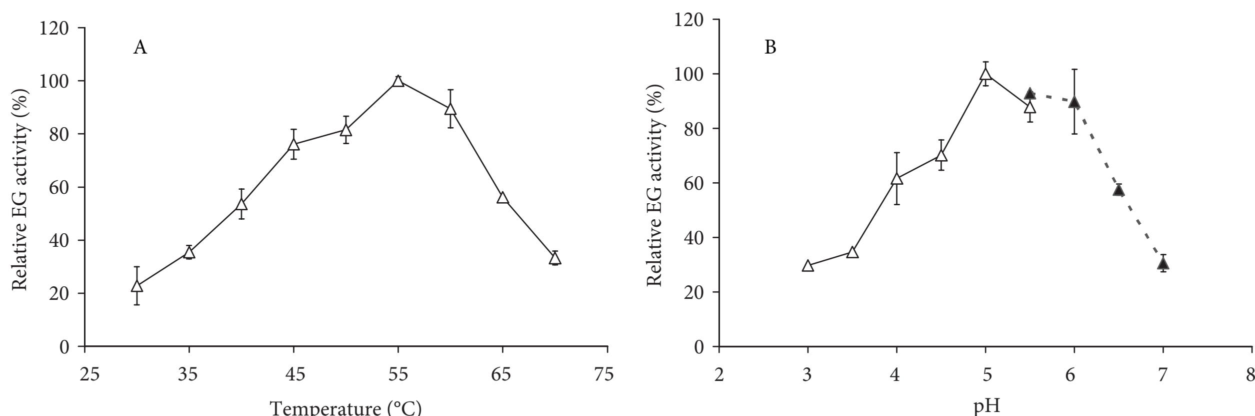

Figure 3. Optimum temperature (A) and pH (B) of EG from *A. niger* VTCC-F021. Relative EG activity was expressed as a percentage of the optimal activity at 55 °C, pH 5 (A) (100% EG activity = 6.801 U/mg) and 45 °C, pH 5 (B) (100% EG activity = 12.079 U/mg).

Temperature and pH stability

A. niger EG was temperature stable up to 50 °C and pH stable in the pH range 5–6; The residual activity was >80% and 60% after incubation at 30–50 °C and pH 5 (Figure 4A) and 37 °C and pH 5–6 (Figure 4B) for 8 h, respectively. Thermostability and pH stability of EG from *A. niger* VTCC-F021 agreed with EG from *A. awamori* VTCC-F099 (8) and *A. oryzae* VTCC-F045 (10). The EG from *A. awamori* VTCC-F099 was temperature stable up to 40 °C and pH stable at pH 4.5–5.5 and retained more than 80% after treatment at 30–40 °C at pH 4.5–5.5 for 36 h (8). The EG from *A. oryzae* VTCC-F045 was temperature stable up to 55 °C and pH stable at pH 5–6; the residual activity increased 21%–27% after treatment at 30–55 °C for 4 h and remained over 72% after treatment at pH 5–6 for 1 h (10).

The EG from *A. terreus* DSM 826 could withstand heating at 50 °C for 1 h without any apparent loss of activity (12). The EG from *A. terreus* M11 was stable at pH 2–5 and retained more than 60% of its activity after heating at 70 °C for 1 h (11). The EG from *A. terreus* strain AN_1 was stable over a broad range of pH (3–5) at 50 °C (23). The EG from *A. niger* IFO31125 was stable at pH 5–10 and showed no loss of original activity after incubation at 60 °C for 2 h (19).

Effect of metal ions and EDTA

The chelating agent EDTA enveloping metal ions showed a 35% increase in EG activity at the 5 mM concentration and a 12% decrease at 15 mM (Table 2), which means the enzyme did not require metal ions for its catalysis. The addition of Cu^{2+} and Fe^{2+} at concentrations of 5–15 mM increased EG activity by 12%–52% (Table 2). The presence of the metal ions K^{+}, Ca^{2+}, Co^{2+}, and Ag^{+} decreased EG activity by 18%–54%. The addition of Mn^{2+}, Zn^{2+}, and Ni^{2+} at 5–10 mM concentrations decreased EG activity to approximately one-third. These results agree with a previous study (10) in which EG from *A. awamori* VTCC-F099 showed enhanced activity, up to 55%, in the presence of Cu^{2+}, Fe^{2+}, and EDTA (10). EG from *A. oryzae* VTCC-F045 was moderately inhibited by the addition of metal ions and EDTA (8).

Chen et al. (2001) reported that the activity of all components of *A. aculeatus* SM-L22 was stimulated by Fe^{2+}. The addition of 25 mM of Co^{2+} and 50 mM of Zn^{2+} activated the EG activity of *A. terreus* DSM 826 by about 83% and 25%, respectively. On the other hand, Hg^{2+} inhibited EG activity by about 50% and 71% at concentrations of 25 mM and 50 mM, respectively (12). The EG activity of *A. terreus* M11 was inhibited by 77% and 59% in the presence of 2 mM of Hg^{2+} and 2 mM of Cu^{2+}, respectively (11). The EG from *A. niger* IFO31125 was inhibited by Hg^{2+} and Cu^{2+} but was not affected by other inhibitors for thiol enzymes such as p-chloromercuribenzoate and N-ethylmaleimide (19).

Cu^{2+} showed a mixed effect on EG activity; it activated the EG from *A. niger* VTCC-F021 (current study) and *A. awamori* VTCC-F099 (10),

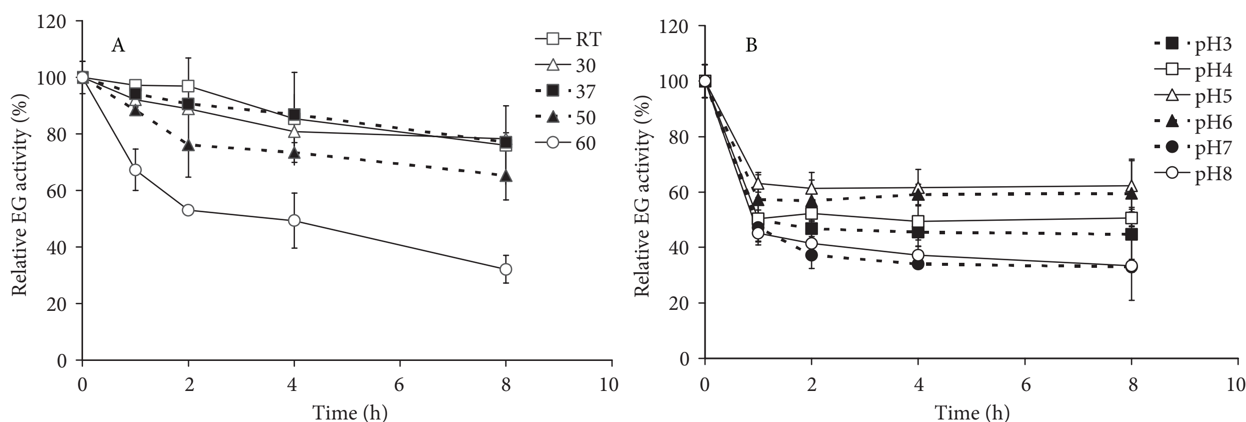

Figure 4. Temperature (A) and pH (B) stability of EG from *A. niger* VTCC-F021. [a] Relative EG activity was expressed as a percentage of the control reaction at time zero (100% EG activity = 10.032 U/mg). [b] Relative EG activity was expressed as a percentage of the control reaction at time zero (100% EG activity = 12.177 U/mg).

Table 2. Effect of metal ions and EDTA on the EG activity from *A. niger* VTCC-F021.

Additive (mM)	Residual EG activity (%) at:		
	5 mM	10 mM	15 mM
Ca^{2+}	85.4 ± 6.4	83.8 ± 12.6	64.0 ± 1.9
Co^{2+}	98.4 ± 3.5	77.7 ± 3.2	62.9 ± 4.2
Cu^{2+}	120.7 ± 2.9	134.2 ± 1.9	152 ± 15.1
Fe^{2+}	129.4 ± 19.7	121.9 ± 5.2	112 ± 6.8
Mn^{2+}	43.9 ± 5.8	41.4 ± 6.1	15 ± 3.5
Ni^{2+}	42.6 ± 18.0	23.0 ± 5.8	12.7 ± 10.6
Zn^{2+}	30.0 ± 10.0	58.8 ± 1.0	26.2 ± 7.1
Ag^{+}	81.8 ± 7.1	72.2 ± 13.5	46.2 ± 8.4
K^{+}	115.0 ± 22.6	96.1 ± 17.1	82.2 ± 12.2
EDTA	135.1 ± 5.2	107.5 ± 6.1	87 ± 19.0
Control	100.0 ± 1.9		

[a] Residual EG activity was expressed as a percentage of the control reaction without any additive (100% EG activity = 10.821 U/mg).

but inhibited EG from *A. terreus* M11 (11), *A. niger* IFO31125 (19), and *A. oryzae* VTCC-F045 (8).

Effect of detergents and organic solvents

The effects of ionic (SDS) and nonionic detergents (Tween 20, Tween 80, and Triton X-100), currently used for denaturing glycoproteins, on EG activity were tested. The EG from *A. niger* VTCC-F021 showed a high resistance to Tween 80 and Tween 20 at 0.5%–2% (w/v) concentrations and retained more than 80% of its initial activity. SDS and Triton X-100 decreased EG activity by two-thirds (Figure 5A). This result agreed with reports on EGs from *A. oryzae* VTCC-F045 (8) and *A. awamori* VTCC-F099 (10), which were highly resistant to Tween 20, Tween 80, and Triton X-100 at concentrations of 0.5%–2.0% (w/v) and Tween 20 at 0.4%–0.8% (w/v), respectively.

Organic solvents are used for solubilizing hydrophobic substrates in enzymatic reactions; thus, we tested the effects of various organic solvents. EG showed a high resistance to methanol and ethanol

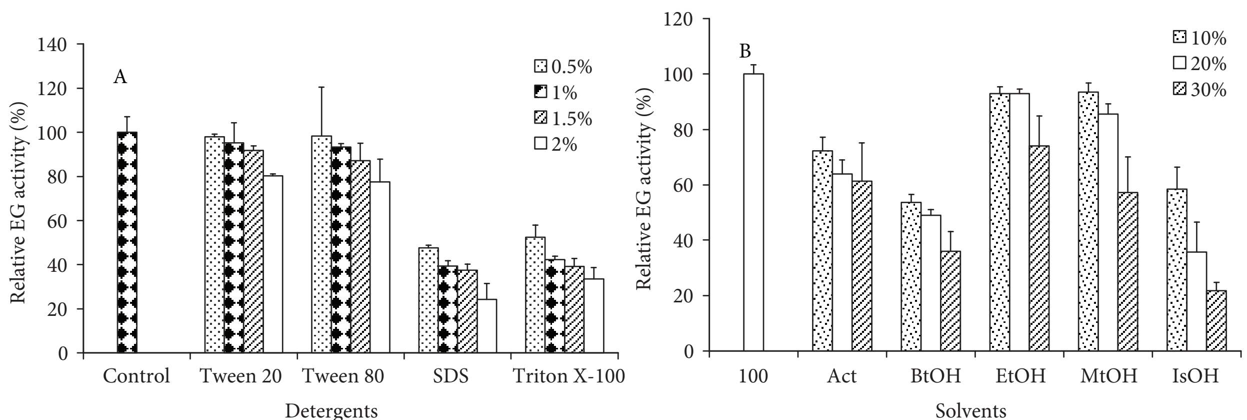

Figure 5. Effect of organic solvents (A) and detergents (B) on EG activity of EG from *A. niger* VTCC-F021. [a] Relative EG activity was expressed as a percentage of the control reaction without any additive (100% EG activity = 8.799 U/mg). [b] Relative EG activity was expressed as a percentage of the control reaction without any additive (100% EG activity = 8.355 U/mg).

at 10%–20% (v/v) concentrations and retained more than 86% of its initial activity (Figure 5B). EG retained also one- to two-thirds of its original activity with the addition of 10%–20% (v/v) acetone, butanol, and isopropanol (Figure 5B). This finding was similar to other reports (8,10), which found that EG from *A. awamori* VTCC-F099 and *A. oryzae* VTCC-F045 was also highly resistant to isopropanol, acetone, methanol, and butanol at 10% (v/v) concentrations and to isopropanol, methanol, and ethanol, respectively, with residual activity of more than 80%.

Conclusions

An extracellular EG from *A. niger* VTCC-F021 was purified to homogeneity, and its properties were investigated. The biochemical properties demonstrated that this EG was quite different from EGs derived from other studied *A. niger* strains. These results suggest that EG from *A. niger* VTCC-F021 could be used to investigate the efficacy of feed enzymes.

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