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EXPERIMENTAL ARTICLES

Kinetic Study of Partially Purified Cellulase Enzyme Produced by *Trichoderma viride* FCBP-142 and Its Hyperactive Mutants[1]

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Abstract—Cellulases are the enzymes that cleave β-1,4 linkages of cellulose, and carbohydrate that is main part of plants' cell walls. Presently, cellulase isolation and partial purification was executed through ammonium sulfate precipitation. The isolated protein of parental and derived mutants conferred molecular weights of 30, 45 and 55 kDa. The optimum temperature for maximal cellulase activity was 50°C with E_a for substrate hydrolysis of 77.73, 83.97 and 83.14 kJ mol^{-1} and temperature quotient of 1.0020, 1.0022 and 1.0022 by *Trichoderma viride* FCBP-142, Tv-UV-5.6 and Tv-Ch-4.3, respectively. The enzyme was stable at 50°C for about 60 min but rapid denaturation occurred above 55°C. The enzyme showed optimum activity at pH 4.0 and involved two types of acidic and basic limbs with pKa_1 and pKa_2. The pKa_1 of active site presented a significant shift from 2.55 to 2.9 and 3.1 by Tv-UV-5.6 and Tv-Ch-4.3, respectively in comparison to parental strain. Likewise, pKa_2 moved from 6.05 to 6.5 and 6.4. Enzyme kinetics displayed Michaelis–Menten constant K_m 0.6, 0.5 and 0.28 mg mL^{-1} and V_{max} value of 8.33, 10 and 9.09 Units mL^{-1} for parental, Tv-UV-5.6 and Tv-Ch-4.3, respectively.

Keywords: Cellulase enzyme, partial purification, *Trichoderma viride* FCBP-142, enzyme kinetics.

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Cellulases are the enzymes which occur in multiple forms and catalyze reactions to participate in the degradation of insoluble cellulose to soluble carbohydrates. These can be used for enhancing drainage rates, deigning, and disposing of cellulosic fines, also used in industries; in the preparation of medicines, resins, perfumes, paper and pulp, in feed treatment, brewing, cereal processing, fruit and juice processing, food fermentation, textile and laundry, detergents, in starch production, waste treatment and baking etc. [1–4]. Keeping in view the great significance of enzymes in wide area of potential applications in various sectors requires purity of this enzyme. Thus, it is essential requisite to develop fiscal processes for the purification of active enzyme [5, 6]. Previously, Becerra et al. [7] compared different enzyme purification techniques to investigate their proficiency, requirement of small amounts of crude extracts as well as the one that allowed enough purified enzyme for several uses. Nagy et al. [8] purified β-galactosidase from *Penicillium chrysogenum* Thom and reported that it is a multimeric enzyme of about 270 kDa with a molecular mass of 66 kDa. The optimum pH and temperature of enzyme activity was 4.0 and 30°C, respectively. Similarly Immanuel et al. [9] worked on the partial purification of the cellulase enzyme from *A. niger* (Van Tieghem) and *A. fumigatus* (Fres.) by SDS–PAGE which revealed that *A. niger* showed two protein bands with the molecular weight of 36 and 23 kDa, respectively. Similarly *A. fumigatus* also had two protein bands with molecular weight of 32 and 21 kDa, respectively. Many other investigators have reported the purification of enzyme from different sources as well as characterization of enzyme [10–15].

Thus the objective of present study was the extraction, partial purification and characterization of cellulase enzyme produced by indigenous strain of *Trichoderma viride* FCBP-142 and its hyperactive mutants Tv-UV-5.6 and Tv-Ch-4.3 to meet the industrial sector demand.

MATERIALS AND METHODS

Isolation and partial purification of enzyme. The selected strains (*Trichoderma viride* FCBP-142 and its hyperactive mutants Tv-UV-5.6 and Tv-Ch-4.3, obtained after UV and chemical mutation, respectively) were grown in Mandel's fermentation medium (Urea 0.3 gL^{-1}, $(NH_4)_2SO_4$ 1.4 gL^{-1}, KH_2PO_4 2.0 gL^{-1}, $CaCl_2$ 0.3 gL^{-1}, $MgSO_4$ 0.3 gL^{-1}, yeast extract 0.25 gL^{-1} and proteose peptone 0.75 gL^{-1} with 10 gL^{-1} of carboxymethyl cellulose) for 5 days on a rotary shaker at 100 rpm and 30 ± 2°C. After 5 days, the broth (crude cellulase) was collected, centrifuged at 7000 rpm for 15 min at 25°C and filtered through a sterilized 0.2 μm Millipore filter to obtain a cell free filtrate (CFF). This

[1] The article is published in the original.

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supernatant or cell free filtrate with enzyme was chilled and precipitated from supernatant with the addition of solid ammonium sulfate from 20 to 100% (w/v) saturation. All the subsequent steps were carried out at 0°C under constant stirring and left for 10–15 min. The aqueous phase was kept overnight at 4°C. The precipitates were formed by binding the protein fraction with ammonium sulfate [16, 17]. The precipitates hence produced were amassed by centrifugation at 7000 rpm for 20 min at 4°C and obtained pallet was dissolved in distilled deionized water and dialyzed against distilled water at room temperature for 24 hours. Diffusion bioassays were performed for the detection of cellulolytic activity.

Quantification and molecular weight determination by SDS–PAGE. Total proteins were estimated according to Bradford method [18]. The OD of different samples was determined at 595 nm wavelength for protein quantification. Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) protocol [17] was adopted for the estimation of molecular weight of partially purified enzyme of selected strains. A protein marker of 11—170 KDa molecular weights was used to pinpoint the desired purified protein. The gel was run at 120 Volts for 14 hours at room temperature. At the same time a native gel was run in the absence of SDS to scrutinize the activity pattern. Entire gel columns of parental as well as mutant derivatives from native gel were separately placed on petri dishes containing 1% soluble cellulose agar and incubated at 30 ± 2°C for 72 hours. Later on, each petri plate was flooded with congo red (0.1% w/v). After sometime the pale clear zone indicated the presence of CMCase activity.

Characterization of partially purified enzyme

Effect of temperature. For the determination of optimum incubation temperature of enzyme, the enzyme was incubated with substrate at various temperatures from 30 to 90°C in 0.05 M citrate buffer for 15 min at pH 4.0 before assaying the cellulase activity. Then energy of activation (E_a) was calculated by using Arrhenius plot as described by Awan [19].

The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate increases due to a raise in the temperature by 10°C. Q_{10} was calculated by rearranging the equation given by Dixon and Webb [20]:

$$Q_{10} = \text{antilog}_e[E \times 10/RT^2],$$

where $E = E_a$ = activation energy.

Thermostability of purified enzyme. Thermal inactivation of native and modified enzyme was determined by incubating the enzyme solutions in 0.05 M citrate buffer (pH 4) at varying temperatures (40, 45, 50, 55, 60, 65, 70, 75°C) in the absence of substrate. Aliquots were withdrawn at different time intervals, cooled on ice for 3 hours and assayed for cellulase activity as described earlier. This procedure was repeated for all the temperatures and data was analyzed.

Effect of pH. The substrate was prepared in 2 buffer solutions: citrate buffer (0.05 M, pH 3.5 to 7.0) and *tris* buffer (0.1 M *tris* HCl, pH 7.5 to 9.0). For the estimation of optimum pH, the enzyme was mixed with substrate at different pH levels (3.5–9.0). Reaction mixture was incubated for 30 min and the activity of purified enzyme was measured for the determination of pK_a's of active site residues as described by Dixon and Webb [20],

Effect of substrate concentration. The effect of various carboxymethyl cellulose concentrations on fixed amount of partially purified enzyme was also studied by using 0.005 to 0.075% substrate solution suspended in buffer. The purified enzyme was assayed at standard assay conditions and Lineweaver–Burk plots were applied as described by Siddiqui et al. [21].

RESULTS

Partial purification of enzyme. Active cellulase enzyme produced by parent strain (*T. viride* FCBP-142) and its mutants (Tv-UV-5.6 and Tv-Ch-4.3) was isolated as cell free supernatant from fermentation medium by using various ammonium sulfate percentage saturations ranging from 20 to 100%. Cell free supernatant subjected to purification exhibited maximum activity of partially purified protein fraction (enzyme) at 70% saturation (Fig. 1). The precipitation of enzyme from parental as well as mutant derivatives revealed almost similar trend. The onset of precipitation was materialized at about 25% saturation while almost complete precipitation occurred at 70% saturation of ammonium sulfate at 0°C. Data acquired on evaluation of purification of cellulase enzyme revealed low activity of pellets recovered below 30% of ammonium sulfate saturation while active pellet/protein concentration was recovered upon stepwise increase from 30 to 70% saturation of ammonium sulfate. The results clearly indicate that the protein concentration was markedly greater in crude samples than in purified protein samples. The overall purification was about 4-fold in parental, as well as UV and chemical mutant strains and the specific activity was found to be substantially increased by purification, in all the test strains (table).

Evaluation of molecular weight by SDS–PAGE. The electrophoretic mobility profiles of known molecular weight proteins were compared with that of partially purified cellulase enzyme of *T. viride* FCBP-142, Tv-UV-5.6 and Tv-Ch-4.3 in 10% SDS–PAGE that exhibited three bands (Fig. 2). The molecular weight assays of these polypeptide protein bands revealed around 30, 45 and 55 kDa values, respectively. These bands (proteins) possibly represent iso-enzymes or different subunits of the same enzyme protein detected on electrophoresis gel. The molecular masses

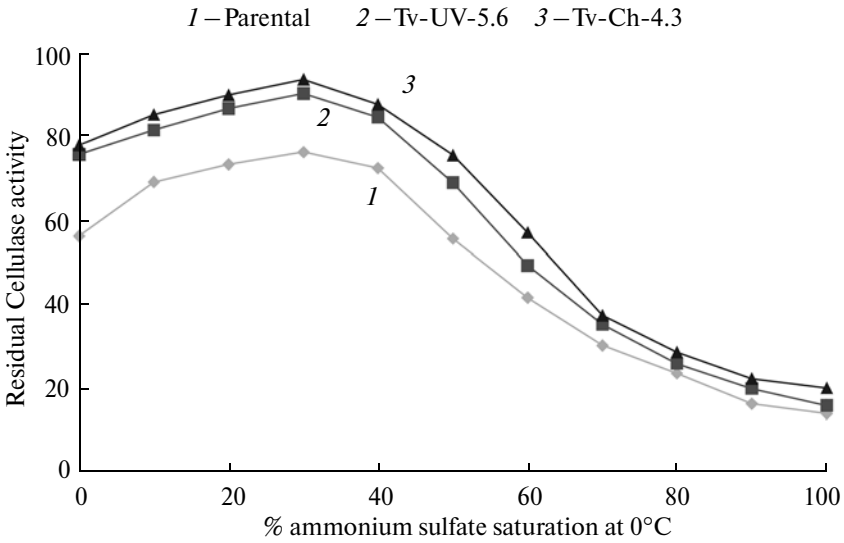

Fig. 1. Ammonium sulfate precipitation of cellulase enzyme from *T. viride 1* – parental FCBP-142, *2* – Tv-UV-5.6 and *3* – Tv-Ch-4.3.

of purified protein determined by native PAGE (without SDS) were almost similar to those evaluated in SDS–PAGE. The presence of enzyme in partially purified protein was further confirmed by placing the sliced samples from each gel column on agar plates and tested through activity staining. The results displayed three bands showing cellulolytic activity corresponding to the band pattern on the stained gel (Fig. 3).

Characterization and kinetics of partially purified enzyme. Partially purified enzyme from parental and mutant strains was further characterized to corroborate the optimum temperature, activation energy, pH and substrate concentrations on the catalytic enzyme reaction. Moreover, extended enzyme stability assays were carried out against various temperature regimes.

Effect of temperature. The effect of temperature on the activity of enzyme was determined in 0.05 M citrate buffer (pH 4.0). The maximum activity was attained at 50°C in wild as well as in mutant strains. At temperatures above and below 50°C, loss in activity was extremely rapid (Fig. 4). The temperature quotient for the enzyme was 1.0020, 1.0022 and 1.0022 for *T. viride* FCBP-142, Tv-UV-5.6 and Tv-Ch-4.3, respectively.

Purification of cellulase enzyme from *T. viride* FCBP-142 and its mutants Tv-UV-5.6 and Tv-Ch-4.3

Strains	Steps	Total Protein (mg)	Total Units (U)	Specific Activity (U mg^{-1})	Purification factor
T. viride FCBP-142	Crude enzyme	358	83.33	0.232	1.00
	$(NH_4)_2SO_4$ Precipitation	74.13	72.44	0.977	4.21
Tv-UV-5.6	Crude enzyme	360.56	107.22	0.297	1.00
	$(NH_4)_2SO_4$ Precipitation	83.05	96.54	1.162	4.00
Tv-Ch-4.3	Crude enzyme	370.58	145.1	0.391	1.00
	$(NH_4)_2SO_4$ Precipitation	85.24	134.8	1.58	4.04

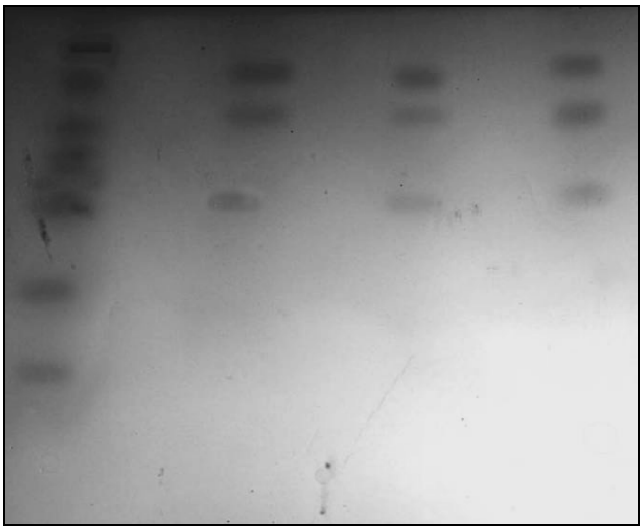

Fig. 2. 10% SDS-PAGE of extracted protein fractions of *T. viride* FCBP-142, Tv-UV-5.6 and Tv-Ch-4.3 to determine the sub units of cellulase enzyme.

The assessments on energy of activation (E_a) of cellulase enzyme for substrate hydrolysis for wild and mutant strains of *T. viride* FCBP-142, was resolved by applying Arrhenius plot. The plots exhibited a biphasic pattern for parent as well as mutant derivatives (Fig. 5). The E_a of cellulase enzyme for substrate hydrolysis by *T. viride* FCBP-142 was 77.73 kJ mol^{-1} which was significantly lower than required by cellulase enzyme from Tv-UV-5.6 and Tv-Ch-4.3 that was around 83.97 and 83.14 kJ mol^{-1}, respectively.

Effect of temperature on stability of enzyme. Thermostability is the ability of enzyme to resist thermal unfolding in the absence of substrates. The results pertaining to thermostability of the purified enzyme are presented in Figs. 6A, 6B. The enzyme was subjected to temperature regimes of 40, 45, 50, 55, 60, 65, 70 and 75°C. The enzyme showed 100% relative activity at 40°C whereas at 45°C some activity was partially lost after 15 min of treatment and onwards. However, at 50°C enzyme activity was reduced to about 40% of the original after 60 min. It was experimentally verified that rapid denaturation occurred above 55°C. The enzyme was evidenced to be thermally very unstable because after heating for only 5 min above 55°C, more than 60% of its activity was lost.

Effect of pH. The cellulase activity was observed to be at its peak in acidic pH regime of 4.0 both in case of wild and mutant derivatives (Fig. 7). Significant inhibition in enzyme activity was detected at pH above and below this optimum pH level. Dixon's analysis was executed to find out ionizable groups of active site residues responsible for maximum velocity of substrate hydrolysis. It revealed that parental cellulase enzyme involved two types of acidic and basic limbs of active

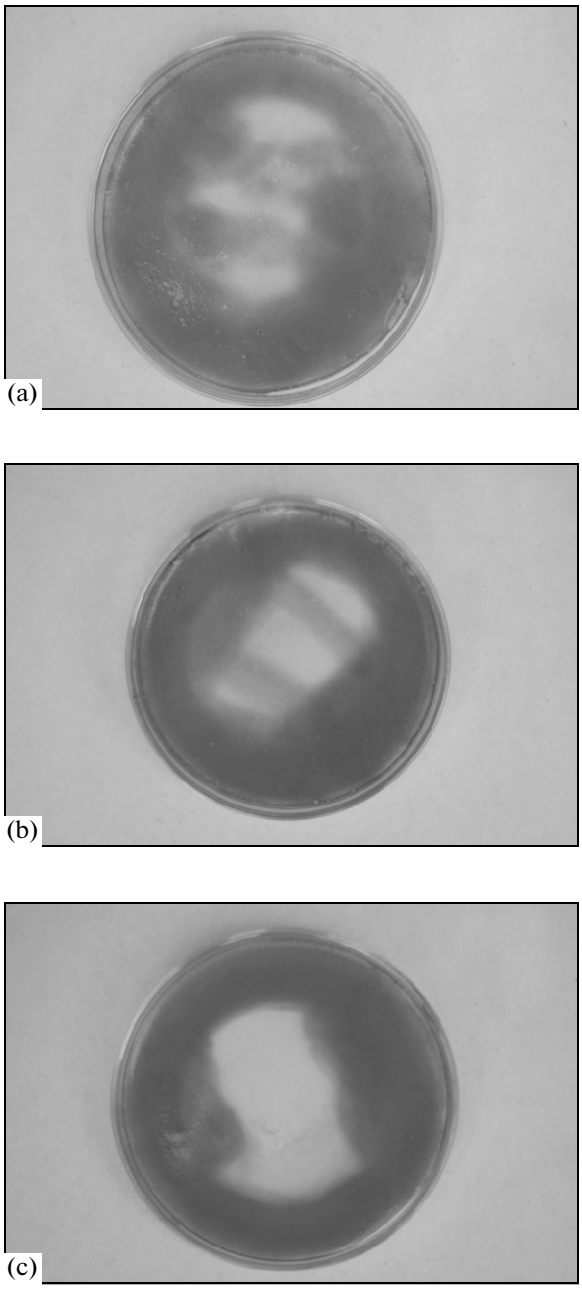

Fig. 3. Bioactivity assay of 10% native PAGE of partially purified cellulase enzyme from *T. viride* FCBP-142, Tv-UV-5.6 and Tv-Ch-4.3 strains.

site residues with pKa_1 and pKa_2 of 2.55 and 6.05, respectively. Due to mutation the conformation of active sites of enzyme was changed. The pKa_1 and pKa_2 values demonstrated by mutant strain Tv-UV-5.6 were 2.9 and 6.5 and by Tv-Ch-4.3 these were 3.1 and 6.4, respectively (Fig. 8).

Effect of substrate concentration. Lineweaver–Burk plot of native *T. viride* FCBP-142 and its mutants Tv-UV-5.6 and Tv-Ch-4.3 was constructed to determine Michaelis–Menten constant (V_{max}, K_m), and findings are presented in Fig. 9. The K_m value for sub-

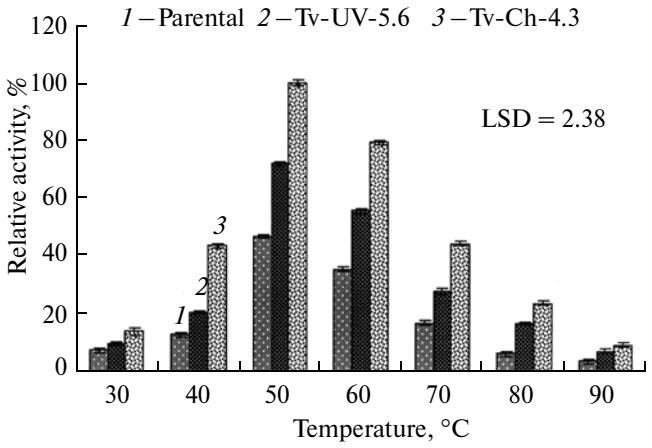

Fig. 4. Effect of temperature on activity of partially purified cellulase enzyme from wild strain of *T. viride* FCBP-142 and mutants Tv-UV-5.6 and Tv-Ch-4.3.

strate hydrolysis by native cellulase enzyme was 0.6 mg mL^{-1}, while Tv-UV-5.6 and Tv-Ch-4.3 exhibited more specificity towards substrate with K_m values of 0.5 and 0.28 mg mL^{-1}. The values of V_{max} illustrated by all the test strains viz., parental, Tv-UV-5.6 and Tv-Ch-4.3 were 8.33, 10 and 9.09 Units mL^{-1}, respectively, which demonstrates that the enzyme produced by mutants was more active than their parent strain.

DISCUSSION

The extra cellular protein was isolated and partially purified from the mass produced test strains through ammonium sulfate precipitation. The isolated protein of parental and derived mutants conferred molecular weights of 30, 45 and 55 kDa. The presence of enzyme in partially purified protein was verified by activity staining. Three bands showing cellulolytic activity corresponding to the band pattern on the stained gel were detected. These proteins may be isoenzymes or different subunits of same enzyme proteins as was reported by Coral et al. [22] and Onsori et al. [13]. A study on the characterization of carboxymethyl cellulase enzyme from wild strain of *Aspergillus niger* Z10 revealed two protein bands from crude enzyme preparations showing cellulolytic activity through SDS–PAGE followed by activity staining of the gel. The molecular weight of these bands was estimated around 83 and 50 kDa [22]. Similar to present records, purification and bioactivity staining has also been reported by many other workers in several previous studies [13, 23, 24].

It is desirable that the activity and stability of enzyme be enhanced. Chemical modification and mutation have been used to enhance thermostability [10, 25, 26]. Melting temperature, thermostability of enzymes at different temperatures, activation energy, effect of pH, proteases and substrates on activity of

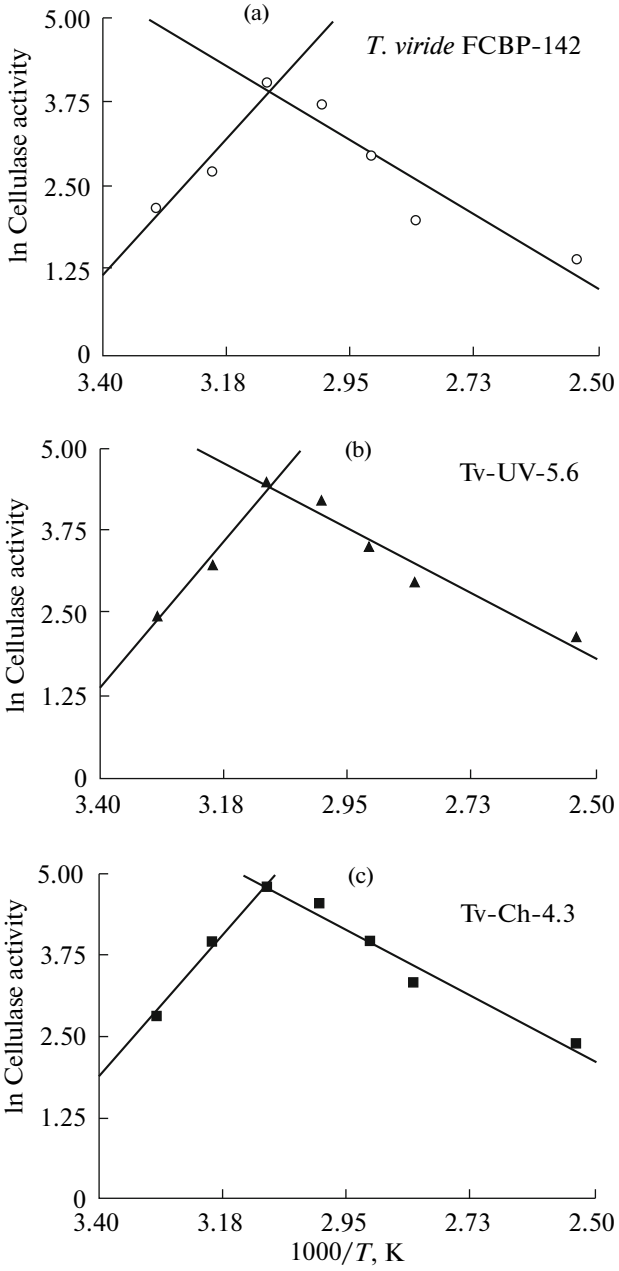

Fig. 5. Arrhenius plot to calculate activation energy of cellulase enzyme produced from wild and mutant strains of *T. viride* FCBP-142 (a), Tv-UV-5.6 (b), and Tv-Ch-4.3 (c).

enzymes are considered potential indices for stability of reactive proteins, however, the intensity as well as the critical point of these parameters was found to diverge with the species or strains used [10, 12]. Presently, kinetic characterization of partially purified cellulase enzyme from the native and mutant strains was performed in order to observe improvement in stability. The productivity decline observed at higher temperatures could be due to the reversible denaturation of enzyme formed on optimized medium as have also been observed previously [27].

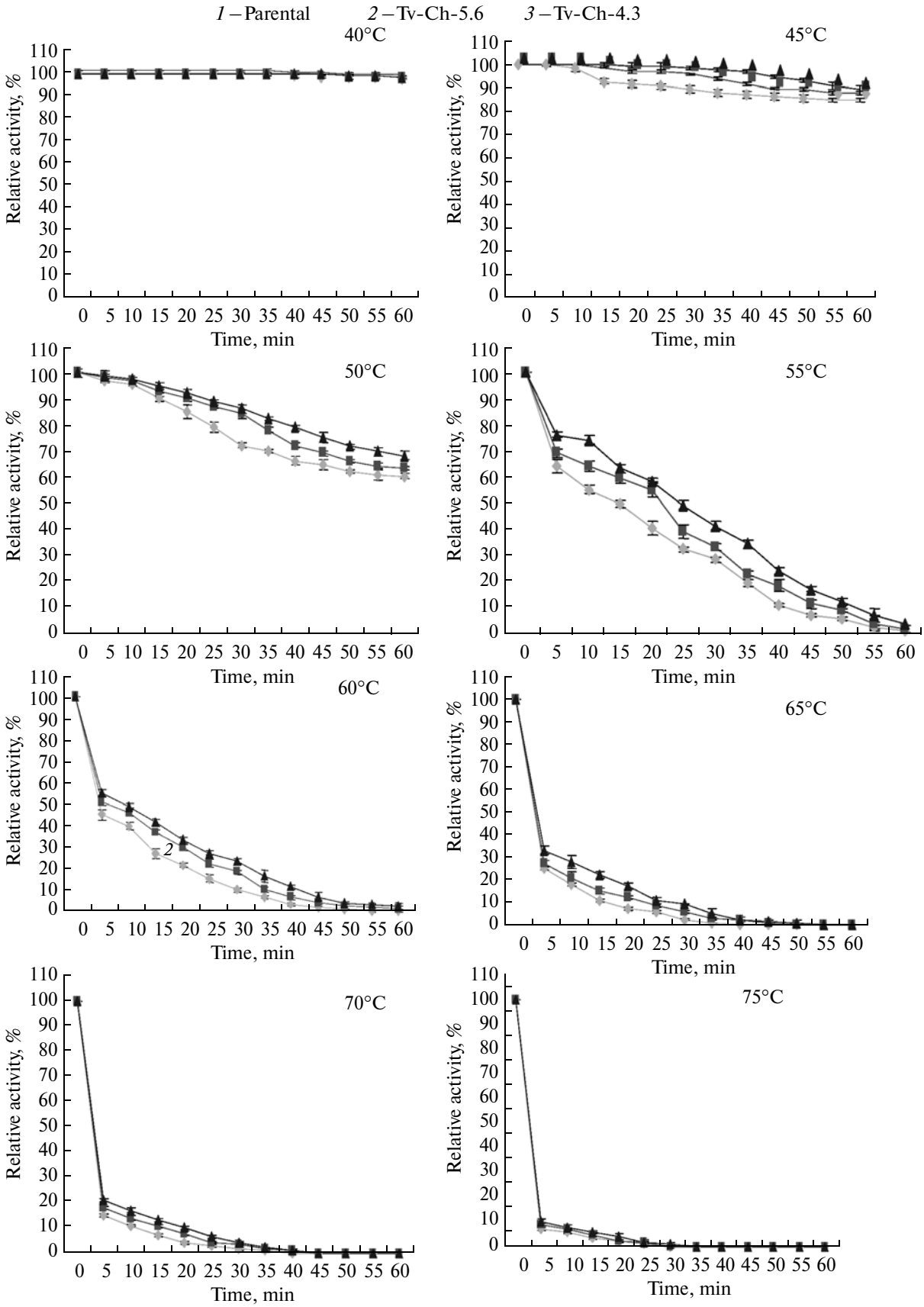

Fig. 6. Irreversible thermostability of partially purified cellulose enzyme from wild strain of *T. viride* FCBP-142 and mutants Tv-UV-5.6 and Tv-Ch-4.3. Vertical bars indicate standart error of means of three replicates. Substrate: 1% cellulose, pH 4.

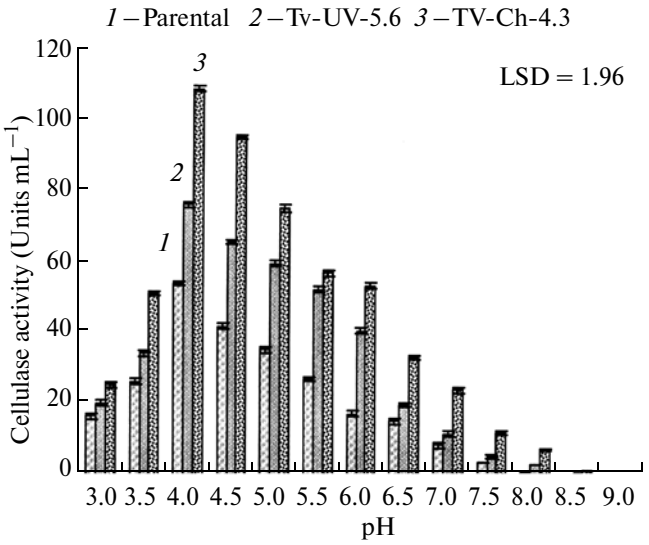

Fig. 7. Effect of pH on activity of partially purified cellulose enzyme from wild strain of *T. viride* FCBP-142 and mutants Tv-UV-5.6 and Tv-Ch-4.3. Vertical bars indicate standart error of means of three replicates. LSO of $P < 0.05$. Substrate: 1% cellulose. Temperature of reaction mixture 50°C.

The enzyme from all the test strains demonstrated optimum activity at pH 4.0. Cellulase enzyme encompassed two types of acidic and basic limbs with pKa_1 and pKa_2. The values of Michaelis–Menten constant K_m were 0.6, 0.5 and 0.28 mg mL^{-1} and V_{max} value of 8.33, 10 and 9.09 Units mL^{-1} were depicted for parental, Tv-UV-5.6 and Tv-Ch-4.3, respectively. Similar findings have been documented by Rajoka and Khan [28]. They stated that partially purified β-xylosidase enzyme from mutant of *Kluyveromyces marxianus* Van der Walt PPY125 showed good stability when incubated at 60°C and at pH 5.0–7.0. Thermodynamic studies revealed that the enzyme derived by the mutant M125 was more thermostable as evidenced by higher midpoint inactivation temperature, lower activation energy demand for β-xyloside hydrolysis.

In another report, xylanase enzymes of three fungi, *Aspergillus indicus* Mehrotra and Agnihotri, *A. flavus* (Link ex Gray) and *A. niveus* Blochwitz, were purified and characterized. The results revealed that the xylanases had optimum pH of 5 to 6 and temperature of 50°C. The enzymes were stable for three days at 37 and at 60°C, *A. indicus* enzyme was stable for 60 min and the other two for 30 min. *A. indicus* xylanase had the highest V_{max} and K_m of 450.20 IU mg^{-1} protein and K_m was 2.50 mg mL^{-1}. *A. flavus*. *A. flavus* xylanase had a lower V_{max} of 383.45 and K_m of 0.8 mg mL^{-1}. *A. niveus* xylanase showed still lower V_{max} values of 313.56 IU mg^{-1} protein but a higher $K_m$of 2.2 mg mg^{-1} , respectively. The molecular weights of the xylanases were 38.7, 36.0 and 32.3 kDa for *A. indicus, A. flavus* and *A. niveus*, respectively, and the corresponding pI values were 4.45, 5.50 and 4.3 [29].

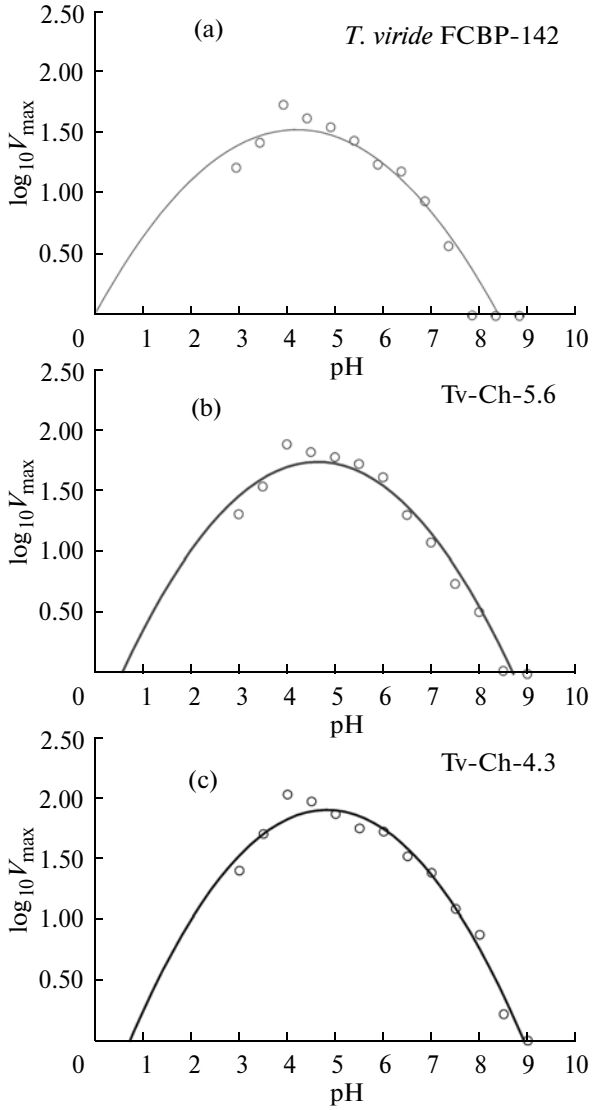

Fig. 8. Dixon plot for the determination of pKa values of active site residues involved in V_{max} of substrate hydrolysis by cellulose from *T. viride* FCBP-142 (a) and mutants Tv-UV-5.6 (b) and Tv-Ch-4.3 (c).

The production and characterization of extra cellular cellulases by a thermoacidophilic fungus, *Aspergillus terreus* Thorn M11, was studied by Gao et al. [30] in solid-state fermentation. Endoglucanase and β-glucosidase exhibited their maximum activity at pH 2 and 3, respectively, with remarkable stability in the range of pH 2–5. The activities of endoglucanase and β-glucosidase were maximum at 70°C and maintained about 65 and 53% of their original activities after incubation at 70°C for 6 h. Many investigators have reported the purification of enzyme from different sources as well as characterization of enzyme [10, 11, 14, 15].

These studies provided insight into enhancement in enzyme activity by mutation and the process of stabilization of enzyme. The mutation induced 2–3 fold increase in enzyme activity and its temperature stabil-

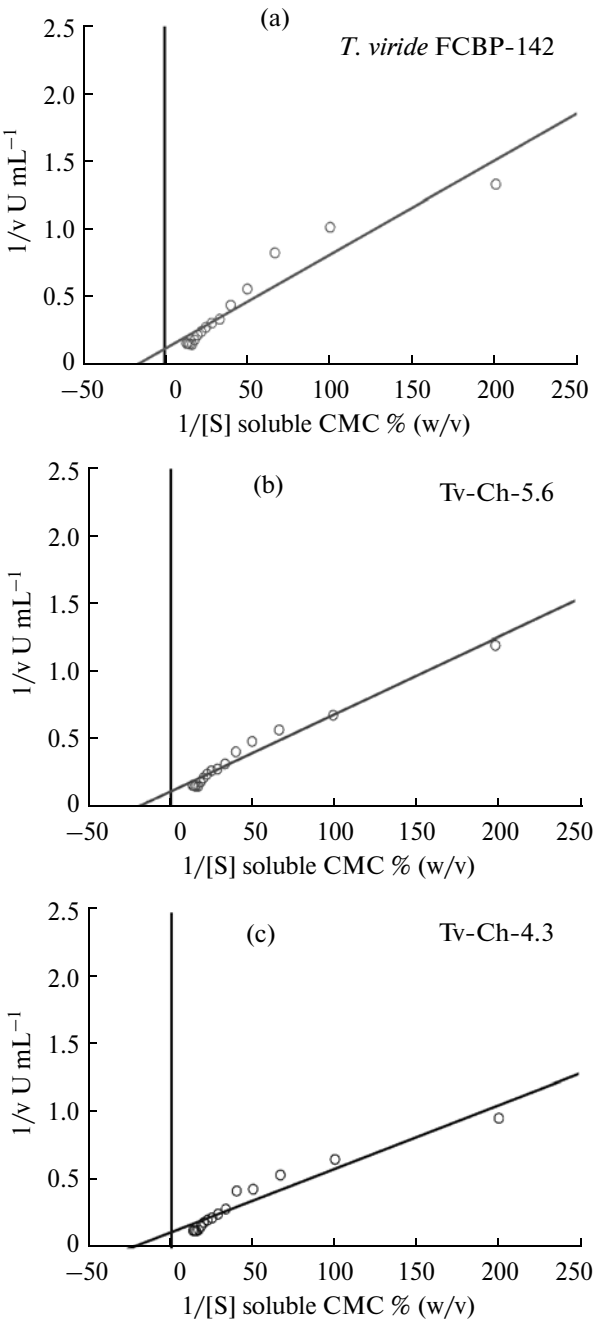

Fig. 9. Lineweaver-Burk plot of partially purified cellulase enzyme from *T. viride* FCBP-142 (a) and mutants Tv-UV-5.6 (b) and Tv-Ch-4.3 (c).

ity was also highly improved. The pKa_1 and pKa_2 values of acidic and basic limbs of enzyme were also altered as a result of mutation exhibiting a change in microenvironment of mutants. The mutant derived enzyme had lower value of K_m in comparison to parent strain indicating less hindrance for substrate binding and declined affinity of the enzyme to active site. Thus these indigenous improved strains can be used for low cost production of enzyme on pilot scale.

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