



Purification and properties of an endoglucanase from *Thermoascus aurantiacus*



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ABSTRACT

An Endo-cellulase was purified to homogeneity using ammonium sulfate precipitation, ion exchange and size exclusion chromatography from newly isolated strain of *Thermoascus aurantiacus* RBB-1. The recovery and purification fold were 13.3% and 6.6, respectively, after size exclusion chromatography. The purified cellulase has a molecular mass (M) of 35 kDa. Optimum temperature for the enzyme was found to be 70 °C and stability was upto 80 °C for 1 h. Along with higher stability at 80 °C, enzyme showed half lives of 192 h and 144 h at 50 and 70 °C respectively. The purified cellulase was optimally active at pH 4.0 and was stable over a broad pH range of 3.0–7.0. The enzyme purified showed apparent K_m and V_{max} values of 37 mg/ml and 82.6 U/min/mg protein respectively with higher salt tolerance of 10% for 1 h.

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1. Introduction

Various types of enzymes are known in the art as additives for detergent compositions. For example, detergent compositions containing proteases, lipases, amylases and cellulases and various combinations thereof have been described in the literature and several such products are currently on the market. Of these enzymes proteases, lipases and amylases are most abundantly used. The enzymes assist in the cleaning of fabrics by degrading their natural substrates protein, fat and starch. Cellulase, on the other hand, is not added to detergent products because of its capability to break down cellulose, but rather to attain certain “care” benefits such as clarification, anti-pilling and reduction of the harshness of the fabric. The harshness reducing action of cellulase has already been described. It is also mentioned that addition of cellulase to a detergent product improves its cleaning performance. Among three different kinds, a particular class endoglucanases have been described as particularly useful for detergent applications.

The majority of commercial cellulases available today are extracellular enzymes produced by mesophilic microorganisms. In response to increasing demand for more stable enzymes,

cellulases from thermophilic microorganisms appear to be ideal for a variety of bioprocesses operating at high temperature [18,34]. Hence new and improved cellulase and related enzymes are necessary to increase the efficiency and economics of various biotechnological processes. Consequently, thermophiles are being aggressively pursued to provide new enzymes that are highly thermostable depending on the environment of the native organism [5].

Present study involves a thermophilic fungus *Thermoascus aurantiacus* [10], which has been known to produce several plant cell wall degrading enzymes including endoglucanases [11,23,36]. It is well known that *T. aurantiacus* is an organism which can produce all three cellulases [1,14,15,20,29]. Specially the major component secreted by *T. aurantiacus*, the endoglucanase exhibits high rates of substrate hydrolysis, superior thermostability, remarkable stability over a wide range of pH values and has tremendous commercial potential [3,31,7]. The endoglucanases find applications in increasing the yield of fruit juices, beer filtration, oil extraction, improving the nutritive quality of bakery products and animal feed, in enhancing the brightness, smoothness, and overall quality of cellulosic garments and in producing fungal protoplast and hybrid strains [3,17].

The quantity of research data described above shows importance of this endoglucanase from *T. aurantiacus*. Hence in the present study, *T. aurantiacus* endoglucanase was analyzed

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in a pure form. The purification was done through ion-exchange and gel permeation chromatography techniques. The study also describes a rapid purification method of the enzyme along with its biochemical characterization.

2. Materials and methods

2.1. Fungal strain isolation and characterization

The strain *T. aurantiacus* studied here was previously isolated from paper pulp industry, Ankleshwar, Gujarat, India [9].

2.1.1. Production of cellulase under solid-state fermentation and enzyme extraction

Cellulase production was carried out in Erlenmeyer flask (250 ml) containing 5 g of the *Jatropha* deoiled seed cake and 2.5 ml of Toyama's mineral medium [19], as the medium with substrate to moisture ratio was [1:0.5]. The medium and substrate were sterilized separately by autoclaving at 121 °C for 15 min. Inoculum of 3 agar plugs of 8 mm diameter was prepared from one week old PDA plates of *T. aurantiacus* was added to the fermentation medium and incubated for 6 days at 50 °C under stationary conditions. The crude enzyme from each flask was extracted using 50 ml of 0.05 M acetate buffer (pH 4.6) and filtered through a wet muslin cloth by squeezing. The filtrate was centrifuged at 8000 × g for 15 min and the clear supernatant thus obtained was stored at –20 °C till further use [9]. The results obtained are average of three independent experiments carried out.

2.1.2. Purification procedure for endo-β-1,4-glucanase from *T. aurantiacus*

The crude protein obtained through fermentation of *Jatropha* deoiled seed cake was subjected for ammonium sulphate precipitation.

2.2. Ammonium sulphate fractionation

Solid ammonium sulphate was added to the culture filtrate to 40% saturation and was precipitated; the resulting precipitate was discarded and supernatant was collected. Again the supernatant was added with calculated amount of ammonium sulphate and kept on stirring. The resulting pellet was obtained through centrifugation at 10,000 × g for 30 min, dissolved in sodium acetate buffer pH 4.6 and dialysed against the same buffer. The dialysed sample was checked for activity of endoglucanase using carboxymethyl cellulose as a substrate and protein content.

2.3. Chromatography techniques for purification of endo-β-1,4-glucanase

A two-step chromatographic procedure was employed to purify endo-β-1,4-glucanase from the crude protein extract. Carboxymethylcellulose was employed as the substrate to monitor carboxymethyl cellulase activity. The experiments were performed at room temperature.

2.3.1. Ion-exchange chromatography on DEAE cellulose DE-52 resin

DE-52, a cellulose based resin, available with GE life sciences was purchased and used for ion exchange chromatography. Approximately 1 ml of ammonium precipitated protein sample was loaded onto an ion-exchange column (Sigma; 10 by 1 cm; 1 ml min⁻¹) previously equilibrated in 50 mM Na-acetate buffer (pH 4.6). The carboxymethyl cellulase was eluted in 50 mM Na-acetate buffer (pH 3.6) with a 0.1–0.5 M NaCl gradient. Fractions

were collected and tested for endo-β-1,4-glucanase activity. Active fractions were pooled and used for further purification of enzyme.

2.3.2. Size exclusion chromatography

The enzyme preparation from ion-exchange chromatography was purified further with size exclusion chromatography. Fractions showing endo-β-1,4-glucanase activity were pooled and loaded onto a Biogel P-100 (10 by 1 cm) column 0.5 ml min⁻¹ previously equilibrated in 50 mM Na-acetate buffer (pH 4.6). Buffer was used as mobile phase to purify the enzyme. Fractions of 200 μl were taken. All the fractions were again checked for endo-β-1,4-glucanase activity. Fractions showing endo-β-1,4-glucanase activity were collected and checked on SDS-PAGE for purity of the protein.

2.4. Enzyme assay

Diluted enzyme solution, 0.5 ml, was mixed with 0.5 ml 2% (w/v) CMC in 0.05 M sodium acetate buffer (pH 4.6) at 60 °C. Reducing sugar content was determined after 15 min by the 3,5-dinitrosalicylic (DNS) reagent method. The reducing sugars were measured according to [30]. One unit (U) of activity was defined as the amount of the enzyme that liberated 1 μmol reducing sugar per minute from the substrate.

2.5. Protein determination

Bradford's method [4], using Bio-Rad assay reagent and bovine serum albumin (BSA) as the standard, were used to determine protein concentration.

2.6. SDS-PAGE

Electrophoresis was performed according to the method described by [25] on 12% polyacrylamide gels. The samples were subjected to SDS-PAGE (resolving gel: 12%; stacking gel: 5%) by using Bio-Rad apparatus, USA. Electrophoresis was performed at room temperature at 60 V till samples migrate into resolving gel and then at 80 V till the dye front reaches towards end of the resolving gel. Along with the protein samples appropriate molecular weight marker was run on gel (Medium range molecular weight marker, Genei, Merck, India). The gels were stained with Coomassie blue R250.

2.7. Non-denaturing PAGE

The purified enzyme was electrophoresed along with marker on 12% (v/v) PAGE containing SDS at 4 °C. Following this, the gel was placed on an agar plate containing 2% CMC (medium viscosity). The plate was incubated at 60 °C in presence of sodium acetate buffer (50 mM, pH 4.6). The gel was stained with 0.1% congo-red and washed with 1 M NaCl to detect enzyme activity [24].

2.8. Characterization of purified enzyme

2.8.1. Effect of temperature on activity and stability of endo-β-1,4-glucanase

The optimum temperature for purified endo-β-1,4-glucanase was obtained by assaying the enzyme activity as described in Section 2.4 at temperature range of 30–100 °C. The results are reported as relative enzyme activity.

The thermo stability for the enzyme was monitored by incubating the enzyme solutions at various temperatures between 30 and 90 °C for 60 min and measuring the activities as described in Section 2.4. The results are reported as residual enzyme activity.

Table 1
Purification procedure of endo- β -1,4-glucanase from *Thermoascus aurantiacus*.

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude	1167	171	6.82	100.0	1
Amm sulphate dialysed	709	46	15.42	60.8	2.3
Ion exchange	224	5.8	38.64	19.2	5.7
Bio-Gel P-100	155	3.46	44.9	13.3	6.6

2.8.2. Measuring half-life of the enzyme

The half life of the endo- β -1,4-glucanase was measured while incubating enzyme solution at 50 °C and 70 °C. At regular intervals the samples were taken and activity was measured. Residual activity was measured calculating 0 min activity as 100%. The time at which the enzyme showed 50% of its original activity, was considered as its half life at given temperature under standard assay conditions.

2.8.3. Effect of pH on activity and stability of endo- β -1,4-glucanase

The effect of pH was studied and activity was determined over the pH range of 2.0–8.0. The following buffer systems were used: citrate buffer (pH 2.0–5.0) and phosphate buffer (pH 6.0–8.0). The enzyme activity was estimated following the procedure described above in Section 2.4 and reported as relative enzyme activity.

To test the pH stability, the purified enzyme was diluted using respective buffers having pH range from pH 3.0 to 8.0 as described above and incubated for 60 min at 50 °C. The enzyme activity was estimated following the procedure described above in Section 2.4 and reported as residual enzyme activity.

2.8.4. Determination of kinetic parameters

The kinetic parameters (V_{max} and K_m) of the cellulase were determined by varying the concentration of carboxymethylcellulose from 10 to 70 mg/ml in 50 mM sodium acetate pH 4.0. The assays were performed with the enzyme, which had been diluted appropriately with 50 mM sodium acetate buffer pH 4.0. The apparent kinetic parameters were determined from double reciprocal plots [27].

2.8.5. Stability of endo- β -1,4-glucanase in presence of NaCl

The purified endo- β -1,4-glucanase was incubated with different concentrations of NaCl (2–10%) for 60 min at 50 °C. The aliquotes were taken after 60 min, enzyme assay was performed with 2% CMC and residual activities were calculated taking control activity as 100% where no NaCl was added.

2.8.6. Stability of endo- β -1,4-glucanase in commercial detergents

The stability of endo- β -1,4-glucanase in the presence of commercial detergents Ariel, Surf Excel, Wheel, Rin and also with SDS was investigated by incubating the enzyme in presence of the detergents (14 mg/ml) at 60 °C [13]. Enzymes activity was measured after 60 min and the residual activity of the enzyme was determined under standard assay conditions taking control activity as 100% where no detergent was added.

3. Results and discussion

3.1. Fungal strain isolation and characterization

The isolate with an efficient cellulase activity obtained from paper pulp recycling mill's soil was further identified by sequencing ITS rDNA region. The isolated fungus was identified as *T. aurantiacus* which has been described earlier [9].

3.1.1. Production of cellulases under solid-state fermentation and enzyme extraction

The crude enzyme preparation was obtained through fermentation of *Jatropha* deoiled seed cake as a substrate. The crude enzyme preparation thus obtained was centrifuged and was transferred to fresh autoclaved tubes and kept at –20 °C till further use.

3.1.2. Purification procedure for endo- β -1,4-glucanase from *T. aurantiacus*

The endoglucanase was purified through fractionation of concentrated, dialysed protein sample using ion-exchange chromatography, which showed a single peak of endo- β -1,4-glucanase activity. Fractions showing highest activities were pooled and used further. Using gel exclusion matrix (Bio-Gel P-100), the enzyme was purified upto homogeneity with 6.6 fold purity and 13.3% yield along with specific activity of 45 U/mg of protein (Table 1).

A single protein band was observed by SDS-PAGE (Fig. 1), indicating that the endo- β -1,4-glucanase had been purified to homogeneity. Purification of extracellular endo- β -1,4-glucanase from *T. aurantiacus* has been reported earlier [28,31]. It is shown that the organism secretes endo- β -1,4-glucanase into the culture medium. The purified endo- β -1,4-glucanase showed specific activity of 45 U/mg which is also in complete agreement with the reports where it was shown that native endoglucanase from *T. aurantiacus* showed 35 U/mg and 49 U/mg of activity [31,28].

3.1.3. SDS-PAGE

Electrophoresis of the enzyme on SDS-PAGE gave a single band with a molecular weight of ~35 kDa (Fig. 1) as the band of purified enzyme lies between 29 and 43 kDa of the marker bands.

Similar results have been reported for the endoglucanase secreted by the same or other *T. aurantiacus* strains, with polymeric

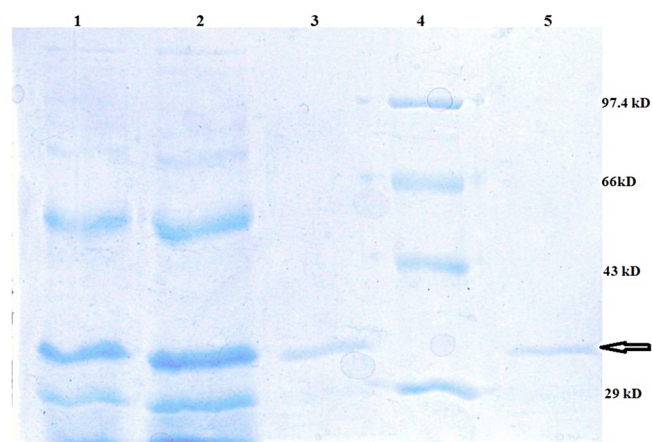


Fig. 1. Purification steps of endo- β -1,4-glucanase. Lane 1: Crude enzyme preparation, Lane 2: Ammonium sulphate dialysed sample, Lane 3: Ion-exchange chromatography fraction, Lane 4: Medium range molecular weight marker (29–97 kDa), Lane 5: Purified protein after Biogel P-100 chromatography.

substrates as carbon sources. When the fungus was grown with wheat straw, paper or corn cobs and molecular weight of the enzyme was estimated by SDS-PAGE at 34.8, 34.0 and 34.7 kDa, respectively [28,31,38].

3.1.4. Nondenaturing PAGE

Lanes: 1: Activity staining with congo red. 2: Corresponding band on PAGE, 3: Medium molecular range marker (14.3–97.4 kDa)

The purified protein was checked for activity on gel. Gel was cut in two parts: one part was used for activity staining and the other was used for development of the gel through Coomassie blue R-250 staining. The results showed that endo- β -1,4-glucanase showed a zone of clearance on agar gel containing CMC (Fig. 2).

Protein of same size showing homogeneity was also observed on the other gel. Again a single band was observed between molecular weight range 29 kDa and 43 kDa. This confirmed successful purification of endo- β -1,4-glucanase (Fig. 2).

3.2. Characterization of endo- β -1,4-glucanase

3.2.1. Effect of temperature on activity of endo- β -1,4-glucanase

The effect of temperature on endo- β -1,4-glucanase activity was examined over a temperature range of 30–100 °C. The activity of endo- β -1,4-glucanase increased gradually with a rise in the temperature up to 70 °C and declined thereafter. The relative activity was low (35%) at 30 °C, which increased to a maximum (100%) at 70 °C and then dropped off to (60%) at 100 °C (Fig. 3).

Thus, this result shows that the optimum temperature for endo- β -1,4-glucanase was 70 °C. It has already been reported that endo- β -1,4-glucanase from *T. aurantiacus* was found to be optimally active at 70 °C [31].

3.2.2. Effect of temperature on stability of endo- β -1,4-glucanase

Utilization of enzymes in industrial processes often encounters the problem of thermal inactivation of the enzyme [35]. Thus, stability of the enzymes is a crucial factor in industrial applications. The stability of endo- β -1,4-glucanase in the temperature range of

30–90 °C revealed the following observations which are elucidated in Fig. S1.

Endo- β -1,4-glucanase was stable up to 70 °C as it exhibited ~100% relative activity. Also, endo- β -1,4-glucanase was found to retain up to 90% activity even after 1 h of incubation at 80 °C. Further increase in the temperature to 90 °C led to a rapid decrease in the stability (~70% activity).

These results are in complete agreement with other studies, which reported that majority of the thermophilic fungal cellulases are stable within the temperature range of 55–80 °C [2,12,22]. It is also been reported that endo- β -1,4-glucanase from *T. aurantiacus* was stable at 70 °C for 8 h [23].

The half life of the purified enzyme was also measured, Enzyme showed half life of 192 h and 144 h at 50 and 70 °C respectively. Earlier studies with the endo- β -1,4-glucanase of *T. aurantiacus* showed that half life of the enzyme was 96 h at 70 °C [14]. The enzyme studied here showed higher stability at elevated temperatures. Enzyme remained stable upto 36 h at 70 °C retaining ~97% of its original activity. The enzyme showed higher stability at 50 °C, it retained about 92% of its original activity after 72 h. It is already been reported by [31], that endoglucanase from *T. aurantiacus* is completely stable for 48 h at 50 °C. The enzyme lost almost its full activity after 210 h of incubation at 50 °C and 192 h of incubation at 70 °C displaying only 26% and 32% of its original activity.

3.2.3. Effect of pH on activity of purified endo- β -1,4-glucanase

Fig. S2 shows that endo- β -1,4-glucanase showed optimum pH at 4.0 as there was a significant increase in activity at pH 4.0. It exhibited ~55% activity up to pH 7.0 and there was a decline in the activity with further increase in pH, it showed 38% of activity at pH 8.0.

Reports also suggest that endo- β -1,4-glucanase is optimally active in the range of pH 4.0–5.5 [8,14,23,26,31,36]. It is well known fact that *T. aurantiacus* produces endoglucanase which is having slightly acidic nature.

3.2.4. Effect of pH on stability of purified endo- β -1,4-glucanase

Enzyme activity is markedly affected by pH because of substrate binding and catalysis is often affected by charge distribution on both, substrate and enzyme molecules [35]. Fig. S3 depicts the pH stability studies in the range of pH 3.0–8.0 and the results obtained show that endo- β -1,4-glucanase was highly stable in the pH range between 3.0 and 7.0 (relative activities \geq 80%).

Stability of endo- β -1,4-glucanase decreased with further increase in pH. Change in pH did not result in a rapid decline in the activity and the enzyme retained ~71% activity at pH 8.0 after

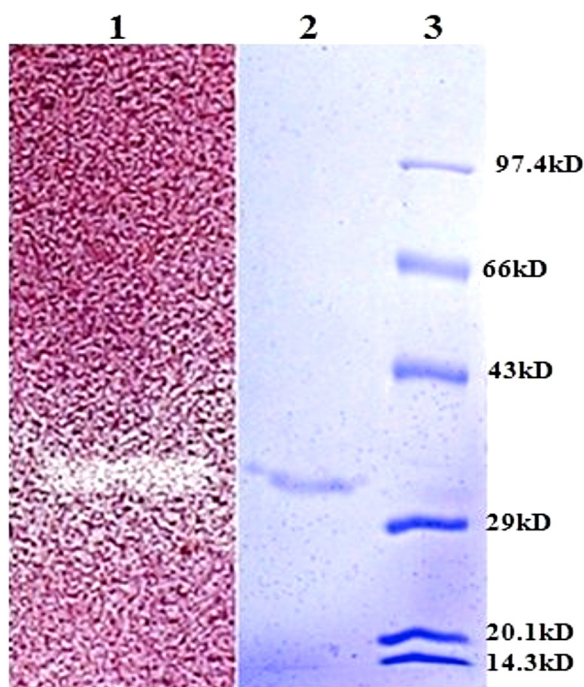


Fig. 2. Activity staining of purified endo- β -1,4-glucanase.

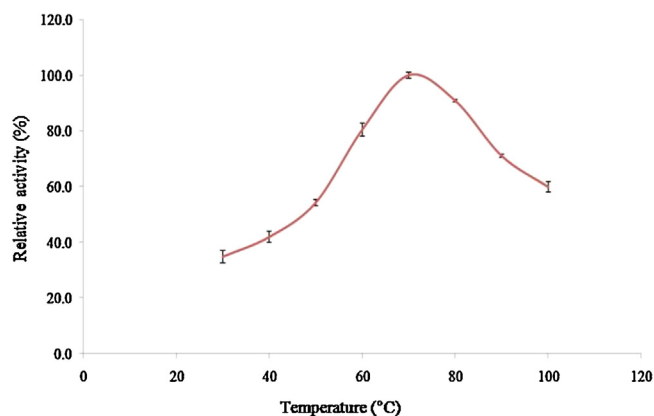


Fig. 3. Effect of temperature on activity of purified endo- β -1,4-glucanase of *T. aurantiacus*. Enzyme activities measured under standard assay conditions and expressed as relative activity of the maximum.

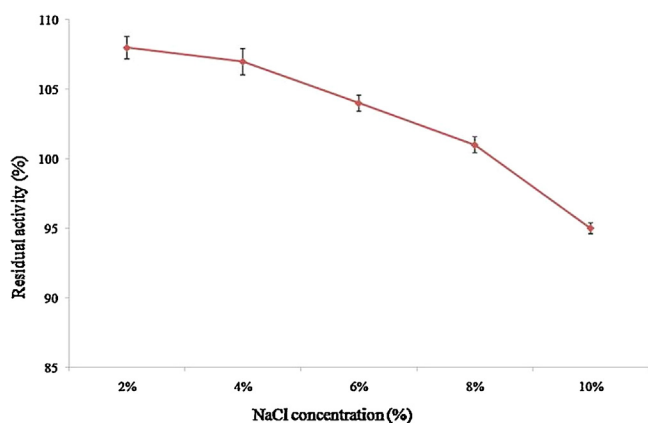


Fig. 4. Effect of NaCl on stability of purified endo-β-1,4-glucanase of *T. aurantiacus*. Enzyme activities measured under standard assay conditions and expressed as residual activity of the maximum.

1 h of incubation. It was also reported that purified endo-β-1,4-glucanase from *T. aurantiacus* was stable in the range of pH 2.8–6.8 [31]. Recently, it was reported that acidic cellulase from *Aspergillus terreus* was found to be stable between pH range 2–5, but the activity of that enzyme dropped after pH 5 [12]. The data shows a promising enzyme property to be used as an industrial enzyme having stability at wide range of pH.

3.2.5. Determination of kinetic parameters

The Michaelis–Menten kinetic constants K_m and V_{max} for purified cellulase were determined by using varying concentration of carboxymethyl cellulose. Enzyme activities were measured under standard assay conditions as described earlier and the catalytic properties, K_m and V_{max} values of purified cellulase from *T. aurantiacus* were 37 mg/ml and 82.6 U/min/mg, respectively using Lineweaver Burk plot.

In literature, different ranges of K_m and V_{max} for different fungal endoglucanase have been reported using carboxymethyl cellulose as a substrate. [33] reported K_m and V_{max} values of 4.8 mg/ml and 72.5 U/mg for an endoglucanase from *Penicillium pinophilum*. A thermostable endoglucanase from *Daldinia eschscholzii* showed K_m and V_{max} values of 1.74 mg/ml and 0.63 U/ml/mg [21]. Another endoglucanase from *Aspergillus awamori* Strain F 18 showed K_m value of 17.24 mg/ml and V_{max} of 28.8 μg glucose/min/IU [37]. [32] also reported one endoglucanase having K_m and V_{max} values 8.5815 mg/ml and 20.121 U/mg.

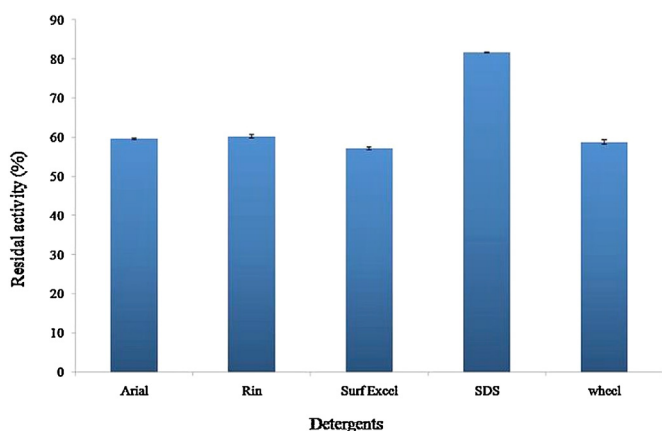


Fig. 5. Effect of commercial detergents on stability of purified endo-β-1,4-glucanase of *T. aurantiacus*. Enzyme activities measured under standard assay conditions and expressed as residual activity of the maximum.

The results obtained show higher substrate requirement for enzyme. The research articles published till date have not shown any K_m and V_{max} value determination for endoglucanase from *T. aurantiacus*, and hence kinetic parameters were compared with other fungal strains.

3.2.6. Stability of endo-β-1,4-glucanase in presence of NaCl

Endo-β-1,4-glucanase was stable upto 8% NaCl for 1 h. Initially in presence of 2–6% NaCl the activity increased and hence the residual activity was higher than the control activity which was calculated as 100%, with increase in concentration of NaCl activity decreased and at 10% NaCl enzyme showed 95% of its activity (Fig. 4). Generally, enzymes from the organisms found in the higher salt containing area are found to be salt tolerant, here as it is shown the endoglucanase showed stability in presence of NaCl concentrations of upto 10% (~95% activity retained) for 1 h.

3.2.7. Stability of endo-β-1,4-glucanase in presence of commercial detergents

There are several reports describing use of endoglucanases from fungi along with detergents to improve activity of the latter. [16] discloses a cellulase preparation consisting essentially of a homogenous endoglucanase which is immune reactive with or homologous to a 34 kDa endoglucanase derived from *Humicola insolens* DSM 1800. Gencore also described use of endoglucanase from *Trichoderma longibrachiatum* in a patent. Lever brother's company used endoglucanase from *Thermomonospora fusca*. Although several of these endoglucanases have been reported to have favorable properties in detergent products, there is still a need to provide alternative or improved endoglucanase containing detergent composition, in particular their stability in liquid detergent formulations [6].

The characteristics of the endoglucanase from *T. aurantiacus* like higher temperature stability and stability of the enzyme in wide range of pH indicates its probable potential for use in detergent formulations.

Fig. 5 describes detergent stability of the purified endoglucanase from *T. aurantiacus*, it showed about 60% of residual activity in presence of all commercial detergents tested. Maximum stability of 62% was displayed by the enzyme in presence of Rin as compared to different commercial detergents. Ariel, Wheel and Surf Excel showed residual activities of 61%, 60% and 59% respectively. Laboratory detergent SDS was found to have less effect on stability of endoglucanase as endoglucanase retained 84% of its original activity after 1 h incubation.

The data obtained showed that enzyme was quite stable in presence of commercial detergents. Enzyme retained about 60% of its original activity in presence of detergents after 1 h incubation. This may be due to its deactivation at higher pH. There are some reports suggesting reduction in stability of enzymes at higher pH values. Activity of *T. longibrachium* endoglucanase having pH optimum of 5.5–6.0, is rapidly decreased in alkaline pH region. The endoglucanase studied here is slightly acidic having pH optima at 4.0. Its pH stability data itself suggest retention of ~71% of original activity after one hour incubation at pH 8.0. So, increased pH value in presence of detergent may led to loss of some activity.

4. Conclusions

The studies carried out suggest that ion exchange and gel permeation chromatography techniques could be effectively used for purification of endoglucanase produced by *T. aurantiacus* on *Jatropha* deoiled seed cake. A ~35 kDa enzyme was purified to homogeneity. Properties like stability at higher temperature for longer period of time and over a wide range of pH along with stability in presence of NaCl and stability in the presence of several

detergents showed that the enzyme can further be used as a potential candidate in various industries.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.btre.2014.11.004>.

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