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Characterization of Exo 1, 4-*β* glucanase produced from *Trichoderma Viridi* through solid-state bio-processing of orange peel waste

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ABSTRACT

Agro-industrial residues are primarily composed of complex polysaccharides that strengthen the microbial growth for the production of industrially important enzymes like cellulases. In the present study we aimed to characterize the Exo 1, 4-*β* glucanase that was indigenously produced from *Trichoderma viride. T. viride* MBL was cultured in the Solid-State medium of orange peel (50% w/w moisture) under optimized fermentation conditions and maximum activity of 412 ± 12 U/mL was recorded after 4th day of incubation at pH 5.5 and 30˚C. Exo 1, 4-*β* glucanase was 4.17-fold purified with specific activity of 642 U/mg in comparison to the crude extract. To confirm its purity and molecular weight, sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) was performed. The enzyme was shown to have a molecular weight of 60 kDa with an optimum pH and temperature of 5˚C and 50˚C, respectively. Lineweaver-Burk reciprocal plot revealed that the kinetic constants K_m and V_{max} of purified Exo 1, 4-*β* glucanase were 76 µM and 240 U/mL.

Keywords: Orange Peel Waste; Exo 1, 4-*β* Glucanase; *T. Viride*; Purification; SDS-PAGE

1. INTRODUCTION

In nature, cellulose, hemicellulose and lignin are the major components of plant cell walls and among all of them, cellulose is the most common and abundant component of all plant matter comprised on about 35% to 50% [1]. It has been reported in literature by many researchers that a wide spectrum of micro-organisms mainly including *Trichoderma*, *Trametes*, *Pleurotus*, *Aspergillus*, *Penicillium*, and *Fusarium* has ability to produce enzymes having industrial importance like cellulases, hemicellulases, lassases, pectinases and proteases [1,2-6]. *Trichoderma* is one of the most competent cellulases producer which is being study extensively for the production of cellulose degrading enzymes from various agro-industrial waste materials and their by-products. Among many of the developing countries it's a routine practice that such agricultural wastes are not been fully discarded that has become a major source of ecological pollution.

From the last several years, there is an increasing demand for industrial important enzymes. In such scenario, cellulase is being used in many of the industrial applications mainly but not limited to in the field of cotton processing; paper recycling, agriculture and in the field of research and development [7-9]. Beside all those applications, the production of fuel ethanol from lignocellulosic biomass through cellulase hydrolysis is a promising tool of the modern world. The most promising technology for the conversion of the lignocellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulase enzymes [10].

Pakistan is an agricultural land that produced abundant magnitude of agro-industrial wastes. However, such wastes can be utilized for the production of useful Industrial enzymes or enzyme based products. Enzymatic hydrolysis of such wastes provides an environmentally friendly means of depolymerizing cellulose and other carbohydrates at high yields [10]. By keeping in mind the ever increasing demand and broad range industrial applications of cellulases, this study was performed to purify and characterize the Exo 1, 4-*β* glucanase from cellulase enzyme complex by *T. viridi* to present its potential application for industrial application.

2. MATERIALS AND METHODS

2.1. Chemicals and Agro-Industrial Substrate

All the chemicals used were of analytical grade and mainly purchased from Sigma-Aldrich (USA). Orange

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peel waste was obtained from the local fruit market, Gujrat, Pakistan and used as a growth supported solid support. Before to use substrate was first crushed into pieces, oven dried and finally ground to fine particle size.

2.2. Fungal Culture and Inoculum Development

Fungal strain *T. viride* was available in the Molecular Biotechnology Laboratory, Department of Biochemistry and Molecular Biology, University of Gujrat, Pakistan. A spore suspension inoculum of *T. viride* was developed in an Erlenmeyer flask containing 30 mL of Potato Dextrose broth at 30˚C ± 1˚C for 5 days after sterilizing at 15 lbs/in^2 pressure and 121˚C.

2.3. Pretreatment of Orange Peel Waste

10 g of moisture free orange peel was pretreated with 2% HCl by adopting thermal treatment methodology as described previously [1]. After pretreatment the slurry of the substrate was filtered using Whatman No. 1 filter paper. Residues were washed 3 times with distilled water to remove extra acidity and used for production of Exo 1, 4-*β* glucanase under optimum fermentation conditions.

2.4. Solid-State Fermentation

For the production of Exo 1, 4-*β* glucanase 10 g pretreated orange peel was moist with Basel salt media in an Erlenmeyer flask (250 mL) capacity. The major constituents of the Basel media were: $(NH_4)_2SO_4$, 10 g·l^{-1}; KH_2PO_4, 4 g·l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g·l^{-1} and $CaCl_2$, 0.5 g·l^{-1}. Orange peel based sterilized Solis-State medium was inoculated with 5 mL of freshly prepared fungal spore suspension and incubated at 30˚C ± 1˚C for stipulated fermentation time period under still culture conditions.

2.5. Extraction of Exo 1, 4-*β* Glucanase

At the end of selected incubation period, Exo 1, 4-*β* glucanase was extracted from the fermented biomass by adding 100 mL of 0.1 M succinate buffer of pH 5 and the flasks were shaken at 120 rpm for 30 min. The contents were filtered and filtrates were centrifuged at 10,000 × g (4˚C) for 10 min. A carefully collected supernatants were and used to determine enzyme activity and for purification purposes.

2.6. Determination of Exo 1, 4-*β* Glucanase Activity and Protein Contents

Exo 1, 4-*β* glucanase was assayed according to the method of Deshpande *et al*. [11], using 1% salicin as reaction substrate with DNS as coupling reagent. The reaction mixture contained 0.1 mL of enzyme extract with 1 mL of 1% salicin and 1 mL of 0.1 M succinate buffer of pH 5. The mixture was incubated for 30 min at 50˚C and the reaction was then terminated by adding DNS reagent (2 mL). The reaction mixtures were heated for 15 min in a boiling water bath followed by cooling in ice. The absorbance was measured at 540 nm against reagent blank. One unit of enzyme activity was defined as the amount of glucose (µmol) released by 1 mL of enzyme solution per min. To determine the protein contents of the crude and purified enzyme extracts bovine serum albumin was used as standard.

2.7. Purification of Exo 1, 4-*β* Glucanase

To purify the crude extract of Exo 1, 4-*β* glucanase obtained from *T. viridi* ammonium sulfate fractionation followed by the Sephadex-G-100 (Sigma, USA) column (120 × 2 cm) gel filtration chromatographic technique was adopted as described by Iqbal *et al*. [1], for purification purposes. Total proteins and activity of partially purified Exo 1, 4-*β* glucanase were determined before and after each purification step as described earlier.

2.8. SDS-PAGE

To determine the molecular weight of purified Exo 1, 4-*β* glucanase sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) was performed on a 5% stacking and a 12% resolving gel according to the methodology, as described previously [1].

2.9. Characterization of Purified Exo 1, 4-*β* Glucanase

Characterization of purified Exo 1, 4-*β* glucanase was done by studying the effect of various kinetic parameters including pH, temperature and substrate concentration on the Exo 1, 4-*β* glucanase activity. To investigate the effect of pH Exo 1, 4-*β* glucanase was incubated in buffers of different pH (2 - 10), followed by standard assay protocol. To determine the thermal features Exo 1, 4-*β* glucanase was incubated without substrate under different temperatures ranging from 25˚C to 70˚C for 1 h time period followed by normal assay protocol as previously described. The Michalis-Menten kinetic constants K_m and V_{max} for Exo 1, 4-*β* glucanase were calculated from Lineweaver-Burk reciprocal plots using varying concentrations of salicin as substrate.

2.10. Statistical Analysis

All the experimental data was conducted in triplicate and presented as mean ± standard error (SE) and SE are showed in figures as Y-error bars.

3. RESULTS AND DISCUSSION

3.1. Production and Purification of Exo 1, 4-*β* Glucanase

T. viride was cultured in Solid-State orange peel based medium for the production of Exo 1, 4-*β* glucanase. Under optimized fermentation conditions in the solid state medium of orange peel (50% w/w moisture) the maximum activity of 412 ± 12 U/mL was recorded after 4th day of incubation at pH 5.5 and 30˚C. *T. viridi* showed high levels of Exo 1, 4-*β* glucanase production during solid state bio-processing of orange peel waste. In the present study, an eco-friendly procedure has been adopted to utilize low cost substrates to induce enzymes production by *T. viridi*. The separated cell free supernatant crude enzyme solution containing Exo 1, 4-*β* glucanase was maximally precipitated at 70% saturation with specific activity of 167 U/mg and 1.98-fold purification. The optimally active fraction was loaded on to a Sephadex G-100 column (**Figure 1**), and after gel filtration the enzyme was purified up to 4.17-fold with specific activity of 642 U/mg (**Table 1**). Previously we have successfully developed and reported Sephadex G-100 column gel filtration technique to purify various fungal enzymes mainly including cellulases from *Trichoderma harzianum* & *Trichoderma viridi*, protease from *Aspergillus niger*, laccase and MnP from *Trametes versicolor* IBL-04 [4-6,12]. In an earlier study, El-Gindy *et al*. [13] has also used the Sephadex-G-100 gel filtration chromatographic technique to purify Exo 1, 4-*β* glucanase produced from *Chaetomiurn olivaceum*.

3.2. SDS-PAGE

Exo 1, 4-*β* glucanase was further purified to homogeneity and to confirm its purity, the purified Exo 1, 4-*β* glucanase was resolved on 5% stacking and 12% running gel and found to be a homogenous monomeric protein as evident by single band of 60 kDa on SDS-PAGE (**Figure 2**). Exo 1, 4-*β* glucanase from *Chaetomiurn olivaceum* was purified to homogeneity by SDS-PAGE with a molecular mass of 88 kDa [13]. While *Talaromyces emersonii* exo-1,3-*β*-glucanase was a monomeric with a molecular mass of 40 kDa [14].

3.3. Characterization of Purified Exo 1, 4-*β* Glucanase

3.3.1. Effect of pH on Exo 1, 4-*β* Glucanase Activity & Stability

The pH-activity profile showed that the present Exo 1, 4-*β* glucanase was optimally activity at a pH 5 (**Figure 3**). It was also observed during the trial that any further increase in the pH showed a sharp decreasing trend in the activity of enzyme. Stability profile based on the 1 h incubation time period revealed that the purified Exo 1, 4-*β* glucanase was stable in a large pH range for up to 1 h. Earlier studies reported that optimum activities of *β*-glucosidase from different enzyme sources in the pH range 5 to 6 [15].

3.3.2. Effect of Temperature on Exo 1, 4-*β* Glucanase Activity and Stability

Figure 4 illustrated that the Exo 1, 4-*β* glucanase from *T. viridi* retained its up to 75% of original activity and was optimally active at temperature up to 60˚C. High activity and extra thermo-stability are a desirable characteristic of an enzyme for a wide range of industrial applications [5,6]. In comparison the earlier reported the present Exo 1, 4-*β* glucanase from *T. viridi* was reasonably more stable and active for up to 1 h incubation at 60˚C. The maximum activity of Exo 1, 4-*β* glucanase was obtained at 45˚C [13].

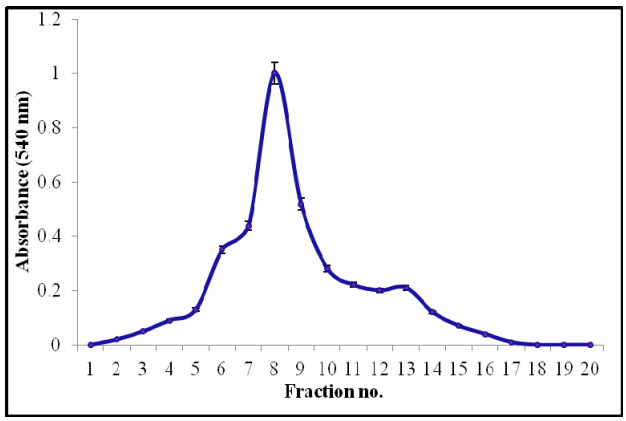

Figure 1. Gel filtration chromatography of Exo 1, 4-*β* glucanase.

Table 1. Purification summary of Exo 1, 4-*β* glucanase pro- duced by *T. viridi*.

Sr. No.	Purification steps	Volume (mL)	Enzyme activity (U)	Protein content (mg)	Specific activity (U/mg)	Purification fold	% Yield
1	Crude Enzyme	200	82,400	535	154	1	100
2	$(NH_4)_2SO_4$ Precipitation	25	10,875	65	167	1.08	13.2
3	Dialysis	20	9600	43	223	1.45	11.7
4	Sephadex-G-100	12	6420	10	642	4.17	7.8

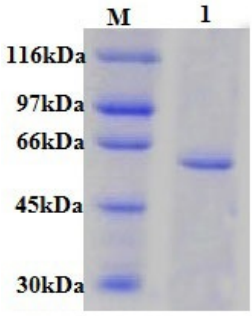

Figure 2. SDS-PAGE of purified Exo 1, 4-*β* glucanase produced from *T. viridi*.

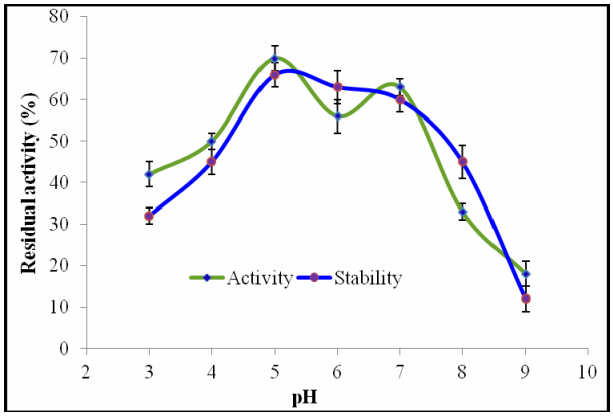

Figure 3. Effect of pH on activity and stability of Exo 1, 4-*β* glucanase.

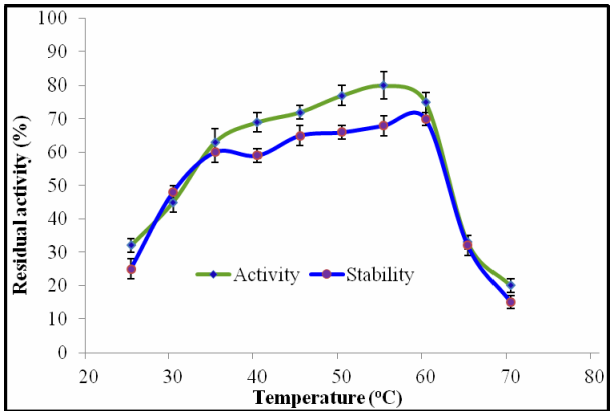

Figure 4. Effect of temperature on activity and stability of Exo 1, 4-*β* glucanase.

3.3.3. Determination of K_m and V_{max}

Using varying concentrations of salicin as substrate, results obtained were plotted as activity against substrate (µM). Lineweaver-Burk double reciprocal plot reflecting substrate affinity and catalytic efficiency of present reported Exo 1, 4-*β* glucanase with K_m (76 µM) and V_{max} (240 U/mL) values as shown in the **Figure 5**. In this article and for the first time the kinetic constants of Exo 1, 4-*β* glucanase through Michalis-Menten transformation Lineweaver-Burk double reciprocal plot has been investigated.

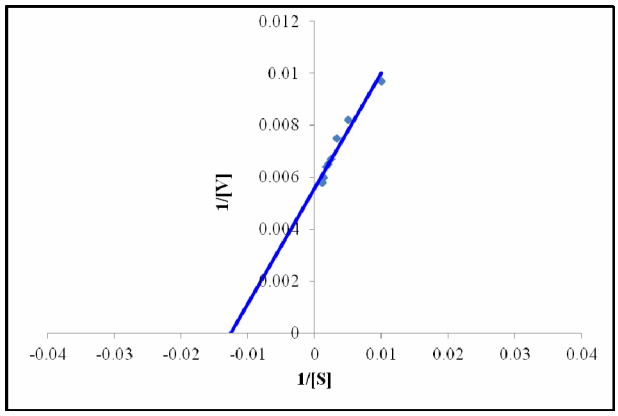

Figure 5. Lineweaver-Burk reciprocal plot: K_m and V_{max} for Exo 1, 4-*β* glucanase.

4. CONCLUSION

T. viridi produces high titers of Exo 1, 4-*β* glucanase during solid state bio-processing of an agro-industrial orange peel waste material. In conclusion, the present reported approach based on the bio-utilization and conversion of agro-industrial orange peel waste into useful products presenting a superior way out for proper waste management for agro based waste materials.

5. ACKNOWLEDGEMENTS

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