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Characterization of purified *β*-glucosidase produced from *Trichoderma viride* through bio-processing of orange peel waste

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ABSTRACT

In the present study, solid state fermentation was carried out using orange peel waste to produce *β*-glucosidase from *Trichoderma viride*. A locally isolated fungal strain *T. viride* was cultured in the solid state medium of orange peel (50% w/w moisture) under optimized fermentation conditions and maximum activity of 515 ± 12.4 U/mL was recorded after 4th day of incubation at pH 5.5 and 30˚C. Indigenously produced *β*-glucosidase was subjected to the ammonium sulfate precipitation and Sephadex-G-100 gel filtration chromatography. In comparison to the crude extract *β*-glucosidase was 5.1-fold purified with specific activity of 758 U/mg. The enzyme was shown to have a relative molecular weight of 62 kDa as evidenced by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The purified *β*-glucosidase displayed 6 and 60˚C as an optimum pH and temperature respectively.

Keywords: Orange Peel Waste; *β*-Glucosidase; *T. viride*; Purification; SDS-PAGE; Characterization

1. INTRODUCTION

The major components of plant cell walls are cellulose, hemicellulose and lignin and among all of them, cellulose is about 35% to 50% which is the most common and most abundant component of all plant matter [1]. Among many of the developing countries, it's a routine practice that such agricultural wastes have not been fully discarded, which has become a major source of pollution. A large variety of micro-organisms including *Trichoderma*, *Aspergillus*, *Penicillium*, and *Fusarium* have the ability to produce enzymes like cellulases, and under mild fermentation environment to hydrolyze insoluble polysaccharides to soluble sugars [1,2]. *Trichoderma* is one of the most efficient cellulases producer organisms which is being studied for the production of cellulose degrading enzymes. Cellulose degrading enzymes system is a complex of three major enzymes that can be divided into three main types: 1) Endoglucanase, 2) Exoglucanases, and 3) *β*-glucosidase [1,3-5].

From the last few years, cellulase is being used in many of the industrial applications, especially in the field of cotton processing; paper recycling, and animal feed additives animal feed industry, agriculture as well as in the field of research and development [4,5]. One of the potential applications of cellulase is the production of fuel ethanol from lignocellulosic biomass. 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 [6,7].

Pakistan is an agricultural land that produced abundant magnitude of agricultural wastes that can be utilized for the production of useful industrial enzymes. Enzymatic hydrolysis of such wastes is one of the attractive solutions of this problematic issue that also provides an environmentally friendly means of depolymerizing cellulose and other carbohydrates at high yields. With respect to the factors affecting culture conditions, productivity and properties of enzymes (cellulase complex), it was considered of significance to purify and characterize this enzyme through kinetic studies to explore such factors. Keeping in mind the broad range of industrial applications of cellulases, this study was performed to purify and characterize the *β*-glucosidase from *T. viridi* to present its potential application for industrial application.

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The present study was also focused on providing a potential solution for the management of large magnitude of solid wastes.

2. MATERIALS AND METHODS

2.1. Agro-Industrial Substrate

Agro-industrial waste orange peel was obtained from local fruit market, Gujrat, Pakistan. The collected substrate crushed into pieces, oven dried and finally ground to fine particle size before to use.

2.2. Fungal Culture and Inoculum Development

The pure culture of *T. viride* was obtained from the Department of Biochemistry University of Gujrat, Pakistan. A homogeneous 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 the potato dextrose broth at 15 lbs/inch^2 pressure and 121˚C for 15 min, and incubated under stationary conditions for the development of fungal spore suspension.

2.3. Pretreatment of Agro-Industrial Waste

10 g of moisture free orange peel was pretreated with 2% HCl by adopting thermal treatment methodology as described earlier [1]. After pretreatment the slurry of substrate was filtered through four layers of muslin cloth, residue were washed 4 to 5 times with distilled water to remove extra acidity and used for production of *β*-glucosidase under optimum fermentation conditions.

2.4. Solid-State Fermentation Strategy

Basel salt media was used to moist the pretreated orange peel in an Erlenmeyer flask for *β*-glucosidase production. The initial pH value of the medium was adjusted to 5 before sterilization at 121˚C and 15.0 lbs/inch^2 pressure for 15 min. The autoclaved medium was inoculated with 5 mL of freshly prepared fungal inoculum and incubated at 30˚C ± 1˚C for stipulated fermentation time period.

2.5. Extraction of *β*-Glucosidase

β-glucosidase was extracted from the fermented biomass by adding citrate buffer 0.05 M of pH 4.8 in 1:10 ratio and the flasks were shaken at 120 rpm for 30 min. The contents were filtered through muslin cloth and filtrates were centrifuged at 10,000 g for 10 min. After that supernatants were carefully collected and used to determine enzyme activity and for purification purposes.

2.6. Determination of Activity & Protein Contents

β-glucosidase activity was determined by the method of Gielkens [8] while, the protein contents of the crude and purified enzyme extracts were determined by following the method of Bradford [9], with Bovine serum albumin as standard.

2.7. Purification and SDS-PAGE of *β*-Glucosidase

Crude extract of *β*-glucosidase obtained from *T. viridi* was centrifuged (10,000 g) for 15 min followed by the ammonium sulfate fractionation as described by Iqbal *et al.* [1]. Total proteins and activity of partially purified *β*-glucosidase were determined before and after dialysis of ammonium sulfate precipitation. *β*-glucosidase was lyophilized and subjected to gel filtration chromatography using Sephadex-G-100 column [10]. The flow rate was maintained at 0.5 mL·min^{-1} and up to 20 active fractions were collected. To determine the molecular weight of purified *β*-glucosidase SDS-PAGE was performed on a 5% stacking and a 12% resolving gel according to the method of Laemmli [11].

2.8. Characterization of Purified *β*-Glucosidase

Characterization of purified *β*-glucosidase was performed to investigate the effect of pH and temperature. *β*-glucosidase was incubated in buffers of different pH (2 - 10), followed by standard assay protocol. To determine the thermal features *β*-glucosidase was incubated under different temperatures ranging from 25˚C to 70˚C for 1 h time period followed by normal assay protocol as previously described.

3. RESULTS AND DISCUSSION

3.1. Production and Purification of *β*-Glucosidase

A locally isolated fungal strain *T. viride* was cultured under optimized fermentation conditions in the solid state medium of orange peel (50% w/w moisture) and maximum activity of 515 ± 12.4 U/mL was recorded after 4th day of incubation at pH 5.5 and 30˚C. *T. viridi* showed high levels of *β*-glucosidase production under SSF as well as growth rate of cells. The eco-friendly procedure has been adopted to utilize low cost substrates to induce enzymes production by *T. viridi*. The separated cell free supernatant as crude enzyme solution containing *β*-glucosidase with activity of 103,000 U/200 mL and specific activity of 149 U/mg was subjected to partial purification by ammonium sulfate precipitation. The crude enzyme was maximally precipitated at 85% saturation with specific activity of 208 U/mg and 1.4 fold purification. The optimally active fraction was loaded on Sephadex G-100 column, and after gel filtration the enzyme was purified up to 5.1 fold with specific activity of

758 U/mg (**Table 1**). Previously Xue *et al.* [12] has also used the Sephadex-G-100 gel filtration chromatographic technique to purify *β*-glucosidase from *R. flaviceps*. *β*-glucosidase was purified by gel filtration on a Sephadex G-100 column [13].

3.2. SDS-PAGE

β-glucosidase was further purified to homogeneity and to confirm its purity, the purified enzyme was resolved on 5% stacking and 12% running gel and found to be a homogenous monomeric protein as evident by single band corresponding to 62 kDa on SDS-PAGE (**Figure 1**). The similarity in the molecular weights determined by denaturing SDS-PAGE suggested that *β*-glucosidase was likely to be monomeric, as reported earlier by Kang *et al.* [14]. Another study, conducted by Xue *et al.* [12] *β*-glucosidase from *R. flaviceps* was purified to homogeneity by SDS-PAGE with a molecular mass of 93.6 kDa while, *β*-glucosidase from *Aspergillus glaucus* (92.5 kDa) [13].

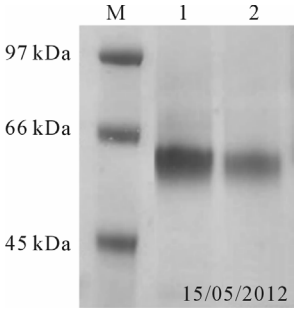
3.3. Characterization of Purified *β*-Glucosidase

3.3.1. Effect of pH on *β*-Glucosidase

The pH-activity profile showed that *β*-glucosidase was optimally active at a pH 6 (**Figure 2**). A further increase in pH showed a sharp decreasing trend. The purified *β*-glucosidase was stable in a large pH range (4.0 to 7.0) for up to 1 h incubation time period. Earlier studies reported optimum activities of *β*-glucosidase from different enzyme sources in the pH range 5 to 6 [15]. Verma *et al.* [16] reported that optimum pH of *β*-glucosidase was in the range of 4.5 to 5.0 while, Xue *et al.* [12] reported that *β*-Glucosidase was stable at pH ranging from 5.0 - 6.8.

3.3.2. Effect of Temperature on *β*-Glucosidase

Figure 3 illustrated that the *β*-glucosidase from *T. viridi* was heat-stable and optimally active up to 60˚C without losing much of its original activity. A wide range of industrial applications required relatively high thermostability as an attractive and desirable characteristic of an enzyme [17,18]. In a recent study Verma *et al.* [16] reported that thermal stability of enzyme *β*-glucosidase was found to be 30˚C while, earlier reported *β*-Glucosi-



[Lane M, Standard protein markers with molecular weights in kDa; Lane 1 & 2, purified *β*-glucosidase]

Figure 1. SDS-PAGE of purified *β*-glucosidase produced from *T. viridi*.

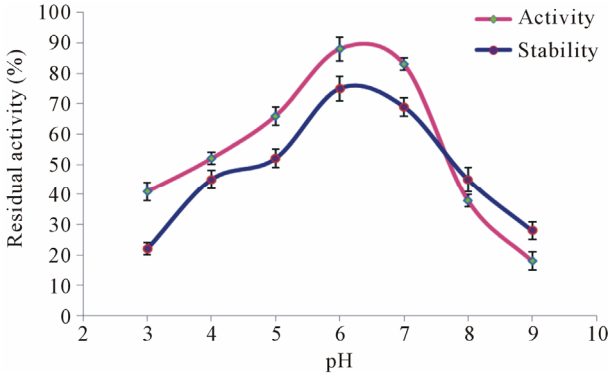


Figure 2. Effect of pH on activity and stability of *β*-glucosidase.

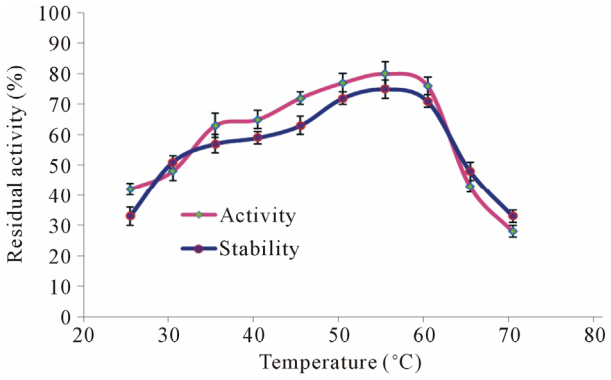


Figure 3. Effect of temperature on activity and stability of *β*-glucosidase.

Table 1. Purification summary of *β*-glucosidase produced by *T. viridi.*

Sr. No.	Purification Steps	Volume (mL)	Enzyme Activity (IU)	Protein Content (mg)	Specific Activity (U/mg)	Purification Fold	% Yield
1	Crude Enzyme	200	103,000	690	149	1	100
2	$(NH_4)_2SO_4$ Precipitation	25	14,375	69	208	1.4	13.9
3	Dialysis	20	12,360	47	263	1.8	12.0
4	Sephadex-G-100	12	8340	11	758	5.1	8.1

dase was stable above 30˚C and below 45˚C. In comparison the earlier reported the present *β*-glucosidase from *T. viridi* was reasonably more stable and active for up to one hour incubation at 60˚C that suggests its potential for industrial applicability.

4. CONCLUSIONS

1) Bio-utilization and conversion of agro are based on waste materials into useful products.

2) *T. viridi* produces high titers of *β*-glucosidase during solid state bio-processing of an agro-industrial orange peel waste material.

3) An extra thermo-stability feature of an indigenous *T. viride β*-glucosidase suggests its potential for industrial applicability and striking prospect for application of this enzyme.

5. ACKNOWLEDGEMENTS

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