RESEARCH ARTICLE

Production and characterization of β -galactosidase from the fungus *Thielaviopsis ethacetica* Went.

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Abstract: β -galactosidase (EC 3.2.1.23) is an industrially important enzyme. It can catalyse two different types of reactions, namely lactose hydrolysis and transgalactosylation. β -galactosidase enzyme production by *Thielaviopsis ethacetica*, was investigated and the enzyme was partially purified and characterized in the present study. Qualitative assay for the pre β -galactosidase activity indicated the formation of water insoluble blue-green colour, dichloro-dibromo-indigo zone towards the edges of the fungal colony after a 72-hour incubation period. Quantitative assay recorded a maximum enzyme activity of 0.599 U/mL at 72 hours. The enzyme was partially purified from the crude enzyme using ammonium sulphate precipitation and dialysis. Fraction 40-60 % (fraction 2) had the highest specific enzymes activity of 1.65 U/mg with 8.68 purification fold and 28.3 % recovery. SDS-PAGE analysis of fraction 2 resulted three separate protein bands above 40 kDa. The protein band corresponding to 50 kDa molecular weight was confirmed as β -galactosidase. The optimum temperature and pH for the enzyme was at 60°C and 7.0, respectively. The present study reveals the fungal strain *Thielaviopsis ethacetica* with β -galactosidase activity. To our knowledge this is the first report about β -galactosidase activity of the strain *T. ethacetica*. This investigation suggests the potential use of this species in industrial applications.

Keywords: β -galactosidase, industrial enzyme, MALDI/MS, starch hydrolysis, *Thielaviopsis ethacetica*.

INTRODUCTION

β -galactosidase (EC 3. 2. 1. 23), also known as β -D-galactohydrolase, β -D-galactoside galactohydrolase,

galactosyltransferase or lactase is an enzyme that has a wide range of applications in the industrial sector. It catalyses two different reactions in organisms: the hydrolyzation of lactose into glucose and galactose (Akcan, 2011), and transgalactosylation reaction which synthesizes important prebiotics such as lactulose and galacto-oligosaccharides (GOS) (Lu *et al.*, 2009). These prebiotics have numerous health benefits and thus β -galactosidase has high demand in both food (Mahoney & Adamchuk, 1980; Jokar & Karbassi, 2009; Princely *et al.*, 2013; Klein *et al.*, 2016; Saqib *et al.*, 2017) and dairy industries (He *et al.*, 2007; Pal *et al.*, 2013; Erich *et al.*, 2015).

β -galactosidases are widely distributed in different biological systems, e.g. microorganisms, plants such as almonds, peaches, apricots, and animal tissues such as brain and placenta (Kayukawa *et al.*, 2020). β -galactosidases are members of the family of hydrolases that belongs to the group of saccharide-converting enzymes. Properties, activity and the structure of the enzyme such as position of the active site, molecular weight of the enzyme, length of the amino acid chain, thermal and pH stability vary with the sources from which the enzyme is obtained (Zhou & Chen, 2001). Therefore, source of the enzyme can be selected according to one's choice, depending on reaction conditions. Among all the natural sources, production of the enzyme is focused on microorganisms as they have the ability to grow on low-cost substrates. Moreover, compared to plants and

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animal sources, higher yields can be obtained while minimizing the cost of production (Santos *et al.*, 1998). Microorganisms including yeast, fungi, bacteria and actinomycetes have been exploited for β -galactosidase production (Zadow, 1992; Fernandez, 1999; Bhanwar & Ganguli, 2014; Panesar *et al.*, 2016; Xu *et al.*, 2019). Among these microorganisms special attention is still paid to fungal sources as their enzymes are thermostable. Since the enzyme is extracellular, additional cost for the extraction is very low. Furthermore, fungi give higher yields, and their enzymes show broad stability profiles. Therefore, the commercially available β -galactosidases have been mainly obtained from fungi: yeast and moulds. The most important sources are *Aspergillus* and *Kluyveromyces* species including *A. oryzae*, *K. lactis*, *K. marxianus* and *K. fragilis* (Zhou & Chen, 2001; Roy & Gupta, 2003; Johnson & Echavarri-Erasun, 2011; Maccaferri *et al.*, 2012; Martarello *et al.*, 2019). As the demand is high, finding new fungal source for the production of the enzyme is of current interest.

The objective of the present study was the optimisation of production, partial purification and characterisation of β -galactosidase secreted by *Thielaviopsis ethacetica*.

METHODOLOGY

The microorganism and culture conditions

Fungal strain *Thielaviopsis ethacetica* has been previously isolated from the topsoil and identified to exhibit alpha-amylase activity (Dissanayaka *et al.*, 2019). The internal transcribed spacer (ITS) sequences have been deposited in the GenBank/NCBI (Accession no; MG062870.1). The fungus was stored in potato dextrose agar (PDA) slants for long term storage and subcultures were prepared once a month throughout the study period. After reviving on PDA supplemented with tetracycline, spores were picked up using a sterile moistened loop and streaked on to 2 % sterile agar plates and incubated at 26 °C for 18 h. Germinated spores were transferred aseptically onto fresh PDA plates to get a single spore culture.

Qualitative assay for the pre β -galactosidase activity

To observe the pre β -galactosidase activity, 6 mm diameter disc of mycelium cut from the growing edge of fungal colonies were transferred aseptically onto YpSs agar plates (2% agar, 0.4% yeast extract, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% KH_2PO_4 and 1.5% soluble starch), supplemented with 80 $\mu\text{g/mL}$ X-gal (5-bromo-4-chloro-

3-indolyl- β -D-galactoside) (Sigma). The plates were incubated at 32 °C for 72 h and the observations were made at every 24-hour intervals. In the presence of β -galactosidase enzyme, X-Gal would be cleaved at the β -1-4 bond between galactose and the 5-bromo-4-chloro-3-indolyl part of X-Gal producing a blue-green colour formation in culture plates.

Determining the time for optimal enzyme production

Pre-inoculum was prepared in 50 mL of fermentation medium comprising 2% soluble starch, 0.5% yeast extract, 0.03% MgSO_4 , 0.03% CaCl_2 and 0.7% KH_2PO_4 at pH 4. Sterile medium was inoculated with 3 agar plugs of profusely growing fungi (6 mm diameter) and incubated at room temperature with shaking at 120 rpm. After 96 hours of incubation, 5 mL of homogenized pre-inoculum (5×10^4 – 6×10^4 spore/mL) was carefully withdrawn and added to 50 mL of the fermentation medium.

Fermentation medium was incubated at 32 °C for 72 h in a rotary shaker at 120 rpm (Dissanayaka *et al.*, 2019). β -galactosidase activity and total protein concentration was measured at 12-hour intervals by removing 150 μL from the fermentation medium. All assays were carried out in triplicate.

β -galactosidase enzyme assay

β -galactosidase activity on soluble starch was assayed by using o-nitrophenol-galactopyranoside (o-NPG) (Miller, 1972).

Appropriately diluted 0.15 mL crude enzyme was mixed with 0.6 mL of substrate solution. 1 mM o-NPG was prepared in 0.1 M sodium phosphate buffer at pH 7.0. The reaction mixture was incubated at 40 °C for 30 min. The reaction was terminated by adding 1 mL of 1 M of Na_2CO_3 . The concentration of o-nitrophenol (oNP) released from o-NPG was determined by measuring the absorbance at 420 nm by UV spectrophotometer using the standard calibration curve (Soydan, 2006; Konsoula & Liakopoulou-Kyriakides, 2007). For enzyme blank, 0.15 mL of sample was mixed with 0.6 mL of buffer and 1 mL of 1 M Na_2CO_3 . For substrate blank, 0.15 mL of buffer was mixed with 0.6 mL of substrate solution and 1 mL of 1 M Na_2CO_3 . Mean absorbance value of the triplicates was taken to calculate the unit enzyme activity. One unit of β -galactosidase activity was defined as the amount of enzyme required to release 1 μmol o-nitrophenol per minute under assay conditions (Soydan, 2006; Konsoula & Liakopoulou-Kyriakides, 2007).

Total protein content assay

Total protein concentration was assayed in triplicate using Qubit 3 Invitrogen™ Qubit™ 3.0 Fluorometer according to the given instructions in Qubit assay kit (Invitrogen™).

Partial purification of the β -galactosidase enzyme

Five hundred millilitres from the submerged fermentation medium containing 2% soluble starch were inoculated with the fungal strain *Thielaviopsis ethacetica* and incubated at 32 °C for 72 h. After the incubation, culture medium was centrifuged at 6000 rpm for 15 min at 40 °C and the supernatant was subjected to gradient ammonium sulphate fractionation. The supernatant was brought into 40% (Fraction 1) saturation followed by 60% (Fraction 2), 80% (Fraction 3), and 100% (Fraction 4). Ammonium sulphate was gradually added into the medium while stirring continuously using a magnetic stirrer (Wingfield, 2001). Saturated protein samples were then centrifuged at 12000 rpm for 15 min, at 40 °C and the protein pellets were recovered. Before the dialysis, each pellet was dissolved in a minimum volume of 0.1 M phosphate buffer at pH 7 separately. Dialysis was accompanied by continuous stirring over 24 hours at 40 °C against the same buffer and the buffer was changed intermittently. Finally, all the fractions were assayed for enzyme activity and total protein concentration.

Investigation the effect of temperature on β -galactosidase activity

To find the effect of temperature and pH on β -galactosidase activity, Fraction 2 (40–60%) with the highest enzyme activity was collected and used. The effect of temperature was evaluated at 20, 37, 40, 60 and 80 °C by incubating the reaction mixture for 30 min. β -galactosidase enzyme activity was determined as previously described.

Investigation of the effect of pH on β -galactosidase activity

The effect of pH on enzyme activity was evaluated by adjusting the pH of the substrate at 4, 5, 6, 7, and 8. The reaction mixture was incubated at 60 °C for 30 min under specific pH value and enzyme activity was determined as previously described.

Determination of the molecular weight of *Thielaviopsis ethacetica* β -galactosidase

Partially purified protein fraction 2 with the highest enzyme activity was freeze-dried (CHRIST Alpha 1-2

LDplus, Germany) and 1 mg of lyophilized protein sample was dissolved in an appropriate volume of sample buffer (Laemmli, 1970). To separate proteins in the sample, 10 % sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) stained with Coomassie Brilliant Blue R-250 was used. To determine the molecular weight of the protein, a broad range molecular weight protein marker ranging from 10–100 kDa was used.

Protein identification using mass spectrometry peptide sequencing (MALDI/MS) method

Molecular weight of many fungal β -galactosidases lies around 40–75 kDa. Therefore, three separated protein bands representing more than 40 kDa were used for MALDI/MS identification. Immediate gel surface around these three bands were carefully separated and stored in an appropriate amount of sterilized distilled water. Three protein samples were sent to ALPHALYSE, Denmark for protein identification using Bruker Autoflex Speed MALDI TOF/TOF instrument. The resulted spectra were searched against available protein databases from UniProtKB using the Mascot software.

RESULTS AND DISCUSSION

Qualitative assay for the pre β -galactosidase activity

Although X-Gal is a commonly associated colourimetric method with lac operon in *E. coli*, the applicability of X-Gal for the detection of fungal β -galactosidase is still under assessment. In the present study, it has been attempted to determine the presence of an active fungal β -galactosidase in culture plates containing X-Gal. IPTG was not added to the culture plates as an inducer. After a 72-hour incubation period, a blue-green colour zone formation was observed towards the edges of the fungal colony. A similar finding has been reported by Fischer *et al.* (1995a), Soydan (2006), and Kishore and Kayastha (2012) with fungal β -galactosidase enzyme. This method will be a great advantage during the early screening procedures when the number of testing samples are high.

β -galactosidase enzyme assay

Thielaviopsis ethacetica containing fermentation medium gave a prominent peak for β -galactosidase enzyme activity during the 3rd day of the incubation. During the first 24 hours protein concentration gradually increased and then peaked at 48 hours (184.2 μ g/mL), where β -galactosidase enzyme activity remained in lower points until 36 h. After 36 hours, enzyme activity

gradually increased reaching its maximum at 72 hours giving 0.599 U/mL maxima with a simultaneous drop in protein content to 67 $\mu\text{g/mL}$ (Figure 1). After 72 hours, enzyme activity remained static where the protein concentration started to increase. Static enzyme activity may probably be due to the denaturation of protein resulted from unfavourable culture conditions during the latter part of the incubation period. Moreover, due to the depletion of initial substrate concentration, newly synthesised mycelia may be secreting other proteins extracellularly to degrade substrates other than starch, resulting in an increased protein concentration after 72 hours.

Figure 1: Total protein content and enzyme activity against incubation time. Error bars represents standard deviation

Optimum β -galactosidase enzyme activity was observed at 72 hours incubation period. Therefore, the

extraction of β -galactosidase from *T. ethacetica* was fixed to 72 hours of incubation period. Late secretion of β -galactosidase enzyme was observed in many other organisms; for example, *Bacillus* sp., *Rhizomucor* sp. and *Aspergillus* sp. (Shaikh et al., 1997; Akcan, 2011; Martarello et al., 2019). Quantitative determination of the β -galactosidase activity is commonly accomplished by using colourimetric method. β -galactosidase is capable of catalysing substrates with b-D-galactopyranoside residues like o-nitrophenol-galactopyranoside (o-NPG). During the hydrolysing process it can form galactose and o-nitrophenol (oNP) with a yellow colour. oNP is capable of absorbing light at 420 nm, therefore the colour intensity can be used to detect β -galactosidase activity of complex biological systems (Miller, 1972).

The present study used starch as the substrate, instead of well-known β -galactosidase substrates containing b-D-galactopyranoside moiety or lactose. The results suggest that one of the intermediate or an end product such as maltose or malto dextrin produced during the starch hydrolysis may act as the substrate for β -galactosidase enzyme production. Konsoula and Liakopoulou-Kyriakides (2007) reported that there can be about 1.5 fold increment of β -galactosidase enzyme activity when using soluble starch as the fermentation medium instead of lactose under the co-production culture conditions of alpha-amylase enzyme. Further, it has been reported that the enzyme production is induced in the presence of yeast extract (Konsoula & Liakopoulou-Kyriakides, 2007). Nitrogen dependency and carbon independency of β -galactosidase have opened many arguments among the scientific community and still there is no strong conclusion regarding its substrate specificity (Lind et al., 1989; Fischer et al., 1995; Konsoula & Liakopoulou-Kyriakides, 2007).

Table 1: Enzyme activity and total protein content of ammonium sulphate fractions

Ammonium sulphate percentage	Total enzyme activity (u/mL)	Total protein content (mg)	Specific activity (u/mg)	Purification fold	Recovery (%)
Crude	8.75	45	0.19	1	100
0–40%	1.15	1.83	0.63	3.31	13.1
40–60%	2.475**	1.5	1.65	8.68	28.3
60–80%	1.776	1.25	1.42	7.47	20.3
80–100%	0.860	0.84	1.02	5.37	9.8

0 – 40% (fraction 1); 40–60 % (fraction 2); 60–80 % (fraction 3); 80–100 % (fraction 4)

**Indicates the highest enzyme activity for fraction 2

Partial purification of the β -galactosidase enzyme

During the partial purification of β -galactosidase, initially, the culture supernatant was saturated with ammonium sulphate from 0–40% (Fraction 1), 40–60% (Fraction 2), 60–80% (Fraction 3) and 80–100% (Fraction 4). Out of the four fractions, fraction 2 had the highest specific enzymes activity of 1.65 U/mg with an 8.68 purification fold and 28.28% recovery (Table 1). Therefore, Fraction 2 was selected for further assay.

Effect of temperature on β -galactosidase activity

Under the defined assay conditions maximum temperature for β -galactosidase enzyme activity was determined at temperatures ranging from 20–80°C. The optimum temperature for maximum *T. ethacetica* β -galactosidase activity was found to be around 60 °C (Figure 2). Many researchers have reported an optimum temperature around 60 °C for *Aspergillus oryzae*, *A. niger*, *A. carbonarius*, *A. fonsecaeus*, *Rhizomucor* sp., *Mucor* sp. and *Fusarium* sp. (Sorensen & Crisan, 1974; Park *et al.*, 1979; Gonzalez & Monsan, 1991; Shaikh *et al.*, 1997; O'Connell & Walsh, 2008; Martarello *et al.*, 2019).

Figure 2: Variation of activity of purified enzyme from fraction 2 with temperature at pH 7.0. Error bars represent standard deviation

Figure 3: Variation of activity of partially purified enzyme from fraction 2 with pH at 60 °C. Error Bars represent standard deviation

Effect of pH on β -galactosidase activity

Thielaviopsis ethacetica exhibited two prominent enzyme activity peaks for β -galactosidases at distinct pH values. The highest activity peak with 0.083 U/mL was observed at pH 7 and slightly lower second peak of 0.054 U/mL at pH 5 (Figure 3).

These results show similarities to the observations made by Fisher *et al.* (1995) for *Thermomyces* sp. In the literature it is reported that most fungal β -galactosidase showed high activity in two pH ranges from 3–5 and 6–7. Borglum and Sternberg (1972), Cardoso *et al.* (2017), Gekas and Lopez-Leiva (1985), Niu *et al.* (2017), and O'Connell and Walsh (2008) reported that most of the *Aspergillus* and *Mucor* species exhibited enhanced enzyme activity under low pH values. On the other hand, Đfrij and Ögel (2002), Fischer *et al.* (1995), Martarello *et al.* (2019) and Matioli *et al.* (2001) reported that the highest enzyme activity was near neutral pH with *Aspergillus*, *Thermomyces* and *Kluyveromyces* species. However, due to having two enzyme activity peaks in the acidic and neutral pH ranges, the present strain can give an additional advantage for use in industry. Considering the acid stability nature of the enzyme, it can be useful in the pharmaceutical industry, while its activity near neutral pH values can be useful in the dairy industry.

Figure 4: Image of SDS-PAGE (fraction 2). Arrows indicate three protein bands used for MALDI/MS identification. (M) molecular marker; (L) protein profile of the *T. ethacetica*

Determination of the molecular weight of *T. ethacetica* β -galactosidase

Partially purified lyophilised fraction 2 was run with a marker protein in SDS-PAGE. The resulted gel image is given in Figure 4. Three proteins bands designated by arrows were used for MALDI-MS/MS identification. MALDI-MS/MS analysis involved 15 partially sequenced peptides and identity searching against UniProtKB using Mascot software. The protein band corresponded to 50 kDa molecular weight was matched to *E. coli* β -galactosidase with 729 Mascot score value (> 95% confidence level). Identified peptides corresponding to *E. coli* β -galactosidase are shown in Figure 5 with 25% sequence coverage. MALDI/MS peptide mapping and sequencing permit the identification of peptide sequences in a mixture especially when it is difficult to obtain one single pure protein. During the SDS-PAGE, Coomassie Brilliant Blue R-250 was used for staining to minimize the interference during mass spectrometry. According to available data on the molecular weight summary report on β -galactosidase, its molecular weight can range from 12 kDa to 270 kDa depending on the species from which it has been isolated. β -galactosidase isolated from *Thermomyces* and *Thermotoganaphthophila* species had molecular weights between 75–80 kDa (Fischer *et al.*, 1995) and 70 kDa (Kong *et al.*, 2014), respectively, whereas the enzyme

1	MTMITDSLAV	VLQRRDWENP	GVTQLNRLAA	HPPFASWRNS	EEARTDRPSQ
51	QIRSLNGEWR	FAWFPAPAEAV	PESWLECDLP	EADTVVVPSP	WQMHGYDAPI
101	YTNVTYPITV	NPPFVPTENP	TSCYSLTFNV	DESWLQEGQT	RIIFDGVNSA
151	FHLWCNGRWV	GYGQDSRLPS	EFDLSAFLRA	GKNRLAVMVL	RWSDGSYLE
201	QDMWRMSGIF	RDVSLHKPT	TQISDFHVAT	RFNDDFSRAV	LEAEVQMCGE
251	LRDYLRTVS	LWQGETQVAS	GTAPFGGEII	DERGGYADRV	TLRLNVENPK
301	LWSAEIPNLY	RAVELHTAD	GTLIEAEACD	VGFREVRLEN	GLLLNGKPL
351	LIRGVNRHEH	HPLHGQVMDE	QTMVQDILLM	KQNNFNAVR	SHYPNHLWY
401	TLCDRYGLYV	VDEANIETHG	MVPMNRLTDD	PRWLPAMSER	VTMRVQRDRN
451	HPSVIIWSLG	NESGHGANHD	ALYRWIKSVD	PSRPVQYEGG	GADTTATDII
501	CPMYARVDED	QFFPAVPKWS	IKKWLSLPGE	TRPLILCEYA	HAMGNSLGGF
551	AKYWQAFRQY	PRLQGGFVWD	WVDQSLIKYD	ENGNPWSAYG	GDFGDTPNDR
601	QFCMNGLVFA	DRTPHPALTE	AKHQQQFFQF	RLSGQTIEVT	SEYLFRRHSDN
651	ELLHWMVALD	GKPLASGEVP	LDVAPQKQL	IELPELPQPE	SAGQLWLTVR
701	VVQPNATAWS	EAGHISAWQQ	WRLAENLSVT	LPAASHAIPH	LTTSEMDFCI
751	ELGNKRWFNF	QSGFSLQMW	IGDKKQLLTP	LRDQFTRAPL	DNDIGVSEAT
801	RIDPNAWVER	WKAAGHYQAE	AALLQCTADT	LADAVLITTA	HAWQHKGKTL
851	FISRKTYRID	GSQMAITVD	VEVASDTPHP	ARIGLNCQLA	QVAERNWLIG
901	LGPQENYPDR	LTAACFDRWD	LPLSDMYTPY	VFPSENGLRC	GTRNLNYGPH
951	QWRGDFQFNI	SRYSQQQLME	TSHRHLHAE	EGTWNLIDGF	HMGIGGDDSW

Figure 5: Partial amino acid sequences of protein band correspond 50 kDa molecular weight of *T. ethacetica* matched to *E. coli* β -galactosidase enzyme. Mapped peptides are shown in bold. 729 Mascot score value (> 95% confidence level).

of *Aspergillus* species reported to have a molecular weight range of 27–126 kDa (Tanaka *et al.*, 1975; Park *et al.*, 1979; Gonzalez & Monsan, 1991; Saad, 2004; Todorova-Balvay *et al.*, 2006). Considering the results of the protein sequencing analysis of the present study, it is indicated that majority of the starch hydrolase at 72 hours represent β -galactosidase enzyme.

CONCLUSION

The present study identified a fungal strain *Thielaviopsis ethacetica* with β -galactosidase activity and to the best of our knowledge this is the first report on β -galactosidase activity by *T. ethacetica*. Enzyme activity at high temperature, pH preference and substrate independency make the β -galactosidase produced by this strain suitable for industrial applications. Further, we emphasize the co-production ability of alpha amylase and β -galactosidase under starch fermentation medium, which can be readily substituted with low-cost substrates such as rice bran, banana peel and cassava starch (Dissanayaka *et al.*, 2019). During the initial stages of growth, *T. ethacetica* secretes extracellular alpha-amylase to the medium and involves starch hydrolysing process. Subsequently, it begins and continues to secrete β -galactosidase up to its death phase. Fungal strains having such beneficial enzyme production characteristics would have a significant impact on industrial-scale applications. Therefore, new opportunities exist for further research on enzyme production abilities of *T. ethacetica* and optimization of such capabilities to fulfil the industrial demands on starch processing.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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