PURIFICATION AND CHARACTERIZATION OF CLONED *PICHIA ETCHELLSII* β-GLUCOSIDASE II AND ITS APPLICATION IN SYNTHESIS OF OLIGOSACCHARIDES

By

YUKTI BHATIA

Department of Biochemical Engineering and Biotechnology

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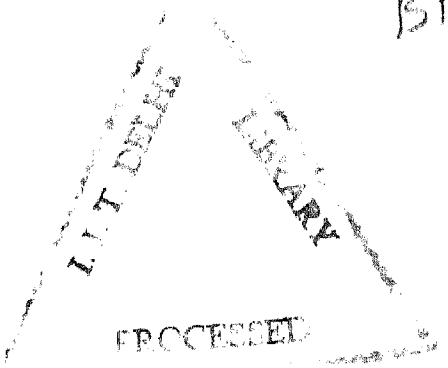

Dedicated to
my sweet and loving parents

CERTIFICATE

This is to certify that the thesis entitled "Purification and characterisation of cloned *Pichia etchellsii* β-glucosidase II and its application in synthesis of oligosaccharides", being submitted by Ms. Yukti Bhatia to the Indian Institute of Technology, New Delhi, for the award of the degree of "Doctor of Philosophy", is a record of the bonafide research carried out by her, which has been prepared under our supervision in conformity with rules and regulations of the "Indian Institute of Technology, Delhi". The research reports and results presented in the thesis have not been submitted for any degree or diploma in any other University or Institute.

Prof. V.S. Bisaria

Prof. Saroj Mishra

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Yukti Bhatia

ABSTRACT

The enzyme β-glucosidase (β-D-glucoside glucohydrolase) catalyzes the hydrolysis of glucosides containing carbohydrate residues linked by β(1→4) linkage. The physiological roles postulated for them are extremely diverse and these are based on hydrolytic as well as synthetic activity of the enzyme. Screening of a number of yeast isolates led to identification of a β-glucosidase producing thermotolerant yeast *Pichia etchellsii*. Based on pH and temperature tolerance, wide substrate specificity and biosynthetic properties exhibited by recombinant BglII (β-glucosidase II) of *Pichia etchellsii* expressed in *Escherichia coli* pBG22:JM109, it was taken up for detailed biochemical and kinetic investigation.

The enzyme BglII was found to be localized in periplasmic space of re-*E. coli*. It was released by osmotic shock and used as starting material for purification. Addition of IPTG led to an increase in specific enzyme activity, indicating partial expression of *βglu2* under *lacZ* promoter. The enzyme was purified 15 fold by using combination of ammonium sulfate precipitation, hydrophobic-interaction and ion-exchange chromatography methods. The biochemical and kinetic properties of purified BglII were investigated in detail. The purified enzyme was found to be a dimer (subunit mass 83 kDa) with an apparent molecular mass of 176 kDa. It was optimally active at 45 ^{0}C and pH 6.0 on *p*NPG. The enzyme also displayed broad substrate specificity and hydrolysed various β-linked disaccharides quite efficiently. Unusually high activity was exhibited on β(1→2) linked sophorose. Kinetic measurements indicated that the best substrate was *p*NPG, with highest value of specificity constant (k_{cat}/K_M) of 1.57 ×10^4 M^{-1}s^{-1}. However, maximum V_{max} value of 1.57 μ mol ml^{-1}min^{-1} was obtained with sophorose. The purified enzyme did not show

any stimulation or inhibition in the presence of divalent ions and EDTA. However, increase in hydrolytic activity was obtained in the presence of lower alcohols like methanol, ethanol, and 1-propanol. Chemical modification studies with group specific modifying agents suggested participation of His residues in catalytic activity. The structural role of Trp residues was also indicated. The purified BglII also showed transglycosylation activity and could synthesize higher oligosaccharides from cellobiose, gentiobiose and sophorose as substrates. Alkyl- and monoterpenyl-glucosides could also be synthesized from cellobiose through transglycosylation reaction.

The biosynthetic reactions of partially purified *Pichia etchellsii* BglII were studied in detail. The effect of incubation time and substrate concentration were determined on the yield of synthesized oligosaccharides. In a reaction time of 24 h, with 16 % (468 mM) initial cellobiose, 22.5 and 6.6 mM of triose and pentaose respectively were synthesized. Addition of dimethyl sulphoxide (DMSO) further increased the yields of the products by 10 %. Detailed kinetic analysis indicated significant (2-fold) increase in V_{max}/K_M of synthetic activity in the presence of DMSO. A study of other disaccharides in transglycosylation reaction indicated biosynthetic activity in the order of sophorose> gentiobiose > cellobiose. A tentative reaction scheme for biosynthesis of oligosaccharides is also presented.

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