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Purification and Characterization of A Novel CGTase from Alkaliphilic *Bacillus* species isolated from Lonar lake , India

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ABSTRACT

Production and purification of Cyclodextrin glycosyltransferase (CGTase) from Alkaliphilic *Bacillus species* isolated from Lonar lake , India was investigated in present study. Production was carried out using medium containing starch, yeast extract, peptone $MgSO_4.7H_2O$. the crude enzyme was collected by centrifugation and partially purified using ammonium sulphate precipitation method. This partially purified enzyme was further purified using phenyl sepharose column chromatography. The enzyme obtained had molecular weight of 79.20 kDa which is confirmed by SDS PAGE and Mass spectroscopy.

Keywords: Cyclodextrin glycosyltransferase (CGTase); purification; Phenyl sepharose; column chromatography.

INTRODUCTION

CDs are non-reducing cyclic structures consisting mainly of 6, 7 or 8 glucose residues, joined by α-(1,4) linkages, for α-, β- and γCD cyclodextrin, respectively. Among the three types of cyclodextrins, β-CD is of high interest due to the size of its non-polar cavity which is suitable to encapsulate several guest molecules; its low solubility in water which facilitates its separation from the reaction mixture. Moreover, β-CD inclusion complexes are easily prepared and more stable (Otero-Espinar *et al*, 2010; Astray et al, 2009).

Alkaliphilic microorganisms have attracted much interest in the past few decades because of their ability to produce extracellular enzymes that are active and stable at high pH values (Atanasova *et al*, 2009; Antranikian *et al*, 2005).

Cyclodextrin glycosyl transferase (CGTase, EC 2.4.1.19) is an important industrial enzyme, unique in its ability to convert starch and related glycans into non-reducing, cyclic malto-oligosacchrarides called cyclodextrins (CDs) via a cyclization reaction, an intramolecular transglycosylation reaction (Biwer *et al*, 2002). Moreover, it is an important hydrolytic enzyme that carries out reversible intermolecular coupling and disproportionation of maltooligosaccharides (Biwer *et al*, 2002; Savergave *et al*, 2008). CGtase is produced by species of *Bacillus, Brevibacterium, Clostridium, Klebsiella, Micrococcus*. The production of CGTase became attractive only when alkalophilic *Bacillus* species were introduced as production organism (Biwer *et al*, 2002; Gawande *et al*, 1999). Various unit operations used in downstream processing for getting pure protein constitute a large part of production cost. Many reports suggest purification strategies of using adsorption of CGTase on starch, which have a drawback that CGTase reacts with starch and a additional step to remove Cyclodextrin is required (Leaver *et al*, 1987). In the present work attempts were made to purify CGTase produced by alkalophilic *Bacillus Species*, using phenyl sepharose column chromatography. And purity of the enzyme was then confirmed by SDS PAGE and Mass spectroscopy by confirming the molecular weight.

MATERIALS AND METHODS

Strain: The strain used for production and purification of CGTase is the isolate isolated from Lonar lake and termed as BI 33, which is identified by 16S rRNA sequencing and according to phylogenic analysis designated as *Bacillus* species and the 16S rRNA sequence was submitted to NCBI GenBank with Accession No. JX419381 (Heydrickx *et al*, 2004).

Production of CGTase using *Bacillus* species:
The production medium used constitute of Solution 1: Starch 1.0 % (w/v), peptone 0.5 % (w/v), yeast extract 0.5 % (w/v), K_2HPO_4 0.1 % (w/v), $MgSO_4$. $7H_2O$ 0.02% (w/v) and Solution 2: Sodium bicarbonate 1% (w/v) (Horikoshi *et al*, 1984)

The organism i.e. *Bacillus* species was inoculated in 510 ml of inoclum medium which is same as that of production medium and enriched. After enrichment 10% of the inoculums was transferred to 100 ml of production medium and incubated at 37°C in rotator shaker incubator for 24 hrs. at the end of incubation period, fermentation medium was centrifuged at 8000 rpm for 10 minutes at 4°C. the supernatant collected was assayed for CGTase activity and was used as crude enzyme for further study (Horikoshi *et al*, 1984).

Cyclization activity of CGTase enzyme:
Cyclization activity of CGTase enzyme sample from isolates *Bacillus* species was determined by the phenolphthalein method (Goel and Nene, 1995). To 1.25 ml of 4.0% soluble starch, 0.25 ml purified CGTase was added. The reaction mixture was incubated for 30 min at 60°C. The reaction was stopped by boiling for 5 min and 1.0 ml of the reaction mixture was incubated with 4.0 ml of phenolphthalein solution. The decrease in phenolphthalein absorption at 550 nm reflected the amount of CD in the reaction which was quantitated from calibration curve. One unit of activity was defined as the amount of enzyme able to produce 1 mole of β-CD per minute under the appropriate condition. Monitoring CGTase activity on agar plate was performed by pouring mixture of methyl orange and phenolphthalein on Horikoshi medium (Park *et al*, 1989) or LB plate in the presence of 1% soluble starch.

Purification of enzyme:
The crude enzyme was used for further purification by ammonium sulphate saturation method, followed by Phenyl sepharose column chromatography.

The crude enzyme was subjected to ammonium sulphate extraction, in this procedure saturated ammonium sulphate solution of different concentration of 30%, 50%, and 70% was prepared. These solutions were mixed with mixture obtained earlier. The protein present in the crude enzyme were allowed to precipitate by keeping the mixture in cold conditions. The precipitate were then centrifuged at 5000 rpm for 20 min, the pellets were collected by dissolving in phosphate buffer of pH 7.0 and was used for further studies (Jogdand *et al*, 2008).

Phenyl sepharose chromatography:
The partially purified enzyme mixture obtained after ammonium sulphate extraction was used for Binding study. Binding study was carried out at different ammonium sulphate concentration from 0.8M to 1.2M. A chromatography column (15X 100 mm size) was packed with phenyl sepharose and equilibrated with 25mM, pH 7.0 Tris-HCl buffer containing 0.8 M to 1.2M $(NH_4)SO_4$, respectively. One ml of concentrated partially purified enzyme mixture was supplemented with 1M

$(NH_4)SO_4$ and loaded to the equilibrated phenyl sepharose column. The elution was carried out by stepwise decrease in the ionic strength of $(NH_4)SO_4$ ranging from 0.25M to 0M. fractions were collected and analysed for CGtase activity and protein concentration (Jogdand *et al*, 2008).

SDS PAGE:

After the partial purification and farction collection, the fraction which shows more CGtase activity and protein concentration was selected and the homogeneity of enzyme in the eluted fraction was checked by SDS - PAGE on a vertical slab gel electrophoresis suing 7.5% acrylamide gel at constatnt current of 30 mA for 2 h. Gel (8cmX 12cm) was run according to the method of laemmli (Laemmli, 1970). The SDS-PAGE was performed, in order to check the homogeneity of the enzyme and to determine the molecular weight of the enzyme under denaturing conditions.

Mass spectroscopy analysis:

The molecular weight and purity was confirmed by mass spectroscopy analysis. The fractions were sent to Department of proteomics, National Chemical Laboratory (NCL), Pune. for mass spectroscopy analysis and the samples were analysed (Nomoto *et al*, 1986).

RESULT AND DISCUSSION

The purpose of this work is the development of simple and effective CGTase purification process immediately from cultural broth. Microorganisms for screening were grown on identical composition medium. The strain producer of CGTase was selected according the highest cyclization provided activity (for 1 ml of cultural broth). Most of the bacterial strains are known to produce other amylolitic enzymes besides CGTases (Volkova *et al*, 2000). Thus the measurement of cyclyzing and dextrinizing activity was conducted during purification process.

Purification of the CGTase:

Usually the gel-filtration is used for CD removal from affinity complex with CGTase (Larsen *et al*, 1998; Bovetto *et al*, 1992). But the pretreatment of applying matter, for instance, concentration and careful calculation, makes this step hard. CGTase from Alkaliphilic *Bacillus* species was purified by ammonium sulphate saturation method followed by phenyl sepharose column chromatography, CGTase was found to be eluted using 0.085 M ammonium sulphate from phenyl sepharose column.

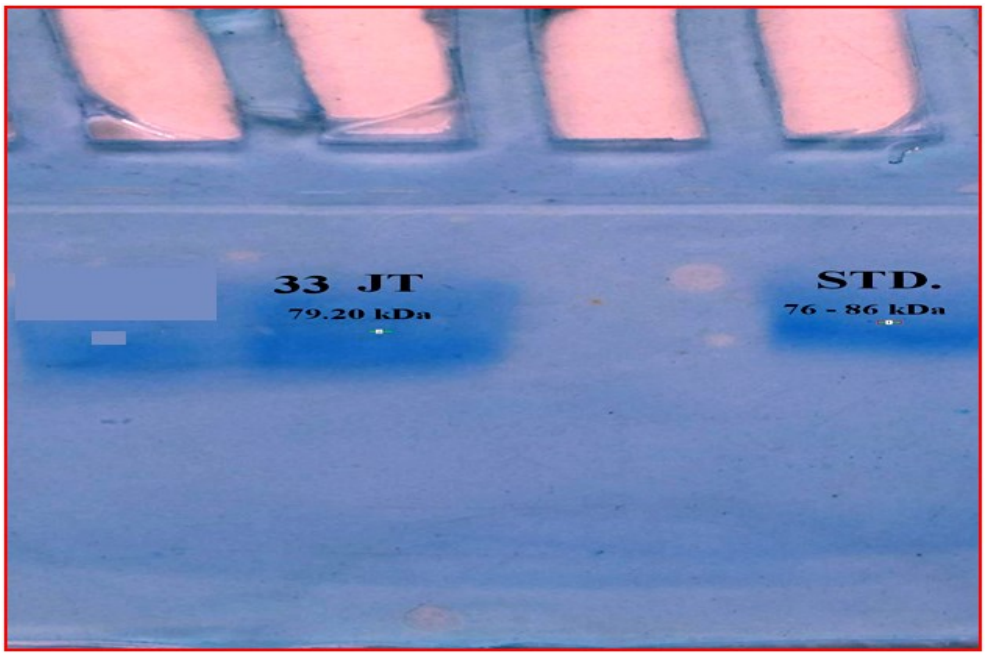

Figure 1: SDS PAGE Analysis Report of CGTase from *Bacillus Species* (33 JT) with Standard CGTase.

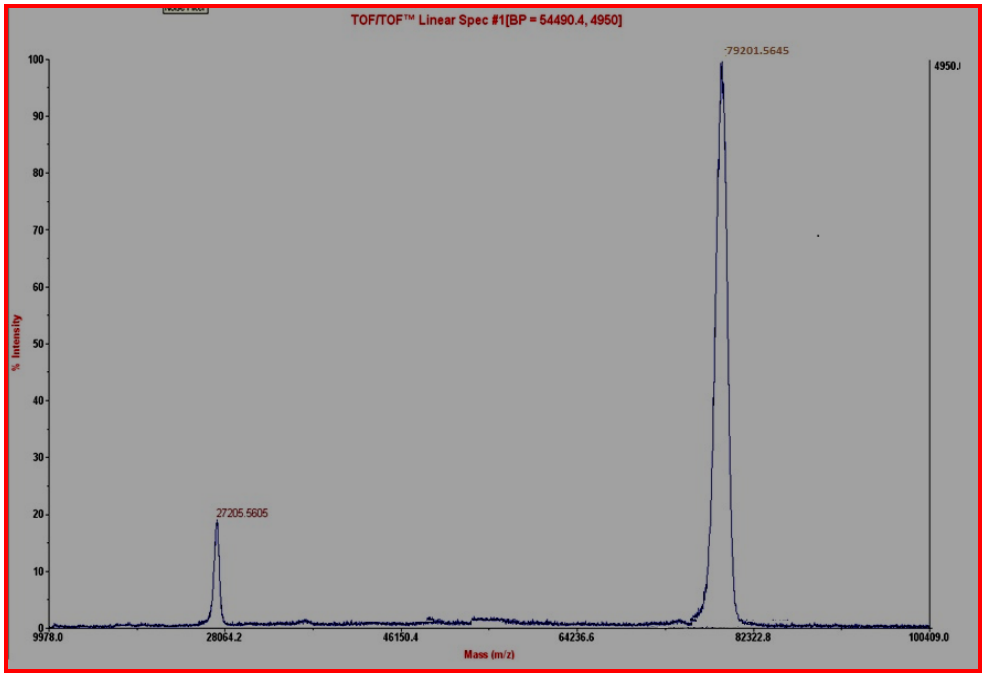

Figure 2: Mass Spectroscopy analysis of partially purified CGTase produced by *Bacillus Species*:

Different separation procedures have been previously applied for obtaining purified CGTases, and in most cases, three or four purification steps were applied including ultrafiltration, gel filtration, starch adsorption and ion exchange chromatography or ammonium sulfate precipitation and two steps ion exchange chromatography (Abdelnasser *et al*, 2012). Yim et al purified the CGTase using DEAE sephadex A-50 followed by DEAE Sepharose CL-6B (Yim *et al*, 1997).

Estimation of Molecular weight of the enzyme:

Some of the physical and chemical properties was identified for purified CGTase. The purified enzyme obtained from phenyl sepharose column showed a single band (Figure 1) by SDS PAGE, which confirms that the homogeneity of the enzyme. Molecular weight of CGTAse was estimated as 79.20 kDa, which is confirmed by Mass spectroscopy (Figure 2). Most of the reported CGTases are monomeric in nature with molecular weight between 60 and 110 kDa. However, CGTases with lower molecular weight have been also reported, such as 33 kDa from *Bacillus coagulans* and as 56 kDa from *Bacillus sphaericus* strain 41 (Abdelnasser *et al*, 2012).

CONCLUSION

In this study, we report purification and characterization of CGTase from Alkaliphilic *Bacillus* species isolated from Lonar lake, India. Enzyme purification to homogeneity was achieved by phenyl sepharose column chromatography. Starch adsorbtion chromatography is one of the popular methods for the initial capture of the CGTase, but it demands gel filteration for the separation of CD formed during the elution of enzyme from the column. Though the rate of purification is slow but we have purified the enzyme successfully without formation of CD in the column. CGTase reported in the study is having molecular weight of 79.20 kDa which is confirmed by SDS PAGE and Mass spectroscopy. Thus, we suggest CGTase purified with the proposed chromatographic scheme is of benefit in comparison to crude enzyme in starch hydrolysis process.

Conflicts of interest: Not declared

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