Trends in Biosciences 11(15), Print : ISSN 0974-8431, 2554-2563, 2018

Evaluation of CGTase Producing *Leucobacter* sp. as a Potential Candidate for Industries

H.S. HAZEENA, R. SUPRIYA, A. JAYAKUMARAN NAIR*

Department of Biotechnology, University of Kerala, Kariavattom, Thiruvananthapuram, Kerala
**email : jekksnair@gmail.com*

ABSTRACT

Cyclodextrin glycosyltransferase (Cyclodextrin gluconyltransferase/ Cyclodextrin glucanotransferase) or CGTase for short (EC 2.4.1.19) is a unique extracellular bacterial enzyme that can degrade starch and related substrate into cyclodextrins (CDs) . In the present study, purification of Cyclodextrin glucanotransferase enzyme from the culture broth of *Leucobacter* sp. isolated from soil was successively carried out by ammonium sulphate fractionation, and purified by DEAE cellulose column chromatography, starch adsorption chromatography and followed by Sephadex gel filtration chromatography. The purified protein obtained existed as only a single band, when assayed by SDS polyacrylamide gel electrophoresis and showed a molecular weight of 64 kDa. The enzyme was later purified upto 98 fold with a 19.5% yield. The purified enzyme was stable over a pH range of 6.5 to 9 showing a maximal activity at pH 6.5. The purified CGTase produced β-CD over a wide temperature range from 40 ^{0}C to 75 ^{0}C with maximal activity at 65 ^{0}C and was found stable at 30 ^{0}C to 65 ^{0}C for a minimum of one hour. The activity of purified CGTase was found enhanced by Mn^{2+} ions but it is strongly inhibited by Zn^{2+} ions. K_m and V_{max} values of the purified enzyme in the reaction mixture were 0.59 mM and 81.5 mM/ minutes of beta cyclodextrin per minute under optimum conditions of enzyme activity.

Key words *β - cyclodextrin, Cyclodextrin glucosyl transferase, CGTase production, purification, thermo stability*

Cyclodextrin glucanotransferases (CGTases; EC 2.4.1.19) are extra cellular enzymes which are able to convert starch and related carbohydrates into cyclodextrins (CDs), a cyclic polymer of glucose (Marlene et al., 2012). It is a multifunctional enzyme that can catalyzes four major and related reactions like cyclizing, coupling, disproportionation, and hydrolysis (Jemli et al., 2007) . They are inducible enzymes produced by microbes . CDs formed by the action of CGTases are cyclic non reducing compounds composed of six, seven or eight glucopyranose residues, classified as α, β, γ – CDs respectively depending on the major CD produced. It is suggested that the CDs produced can be used as a substrate by its producer.

Due to their increasing industrial demands, considerable interest has been focused on the production of CDs by the CGTase activity of microorganisms. CGTases differ in the amount and type of CDs produced. Cyclodextrins are natural cyclic oligo saccharides with dough nut –shaped structure possessing hydrophilic surface and hydrophobic central cavity. Due to this extra ordinary structure CDs are able to form inclusion complexes with different guest, organic and inorganic molecules (Abdel et al., 2007), and as a result it alters the chemical and physical properties of the complexed guest molecules. CDs are widely used in food industry for its stabilizing and increased solubility features, agricultural industry, pharmaceutical, cosmetic and other industries. They are widely exploited for imparting reduced volatility, improved control of the release of drugs and for masking odours and tastes (Hamoudi et al., 2011; Marcon et al., 2009; Wang et al., 2011). The purification, characterization and production of new CGTases attract a great attention due to its increased industrial demand. In this scenario, the alkalophilic bacterium is increasingly exploited due to its increased bio activity over a wide range of temperature and pH. The main aim of this study was to purify CGTase from alkalophilic *Leucobacter* sp. isolated from soil and also to characterize some important properties of the purified enzyme.

MATERIALS AND METHODS

Materials

All chemicals used for the preparation of the buffers, enzyme and protein assay were of analytical grade (M/S Sigma chemicals, USA.). Sephadex G-100, β – CD and Coomassie Blue for SDS-PAGE were purchased from Merck company . Spectrophotometric assays were carried out in U-V spectrophotometer (M/S Shimadzu, JAPAN).

Methods

Bacterial strain and culturing conditions

The bacterial strain was isolated from the soil samples collected from Parambikulum wild life sanctuary and forest area in Kerala. The isolates were grown in Horikoshi media consisting of 1.0% soluble starch(w/v), 0.05% yeast extract, 0.5% peptone, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.03% phenolphthalein, 0.01% Methyl orange, 1% Na_2CO_3 and 1.5% agar (for solid media) and pH was adjusted to 10.5 (Sian et al., 2005). Culture plates were incubated at 37 °C for 72 hours. Horikoshi phenolphthalein (PHP) plate was used

for detecting the CGTase producing organisms. The CGTase activity of the microorganism is identified by the formation of yellowish halo zones around the colonies. From the plate, the bacterial colony with biggest halo zone was selected and streaked on Horikoshi (II) medium for several times to obtain uniform colonies. One strain was identified as a potent CGTase producer and this strain was identified microscopically and biochemically.

CGTase production and purification

The selected organism identified to be a producer of CGTase from phenolphthalin plate was inoculated to 100 ml of Horikoshi broth of pH 10.5 in 500ml Erlenmeyer flask. The media was inoculated with 1 ml of 5 % (v/v) of bacterial inoculum of 0.5 OD at 660 nm. The bacterial culture was incubated at 35°C in an incubator shaker for 6 days. Samples were withdrawn at specific intervals of time, centrifuged at 5000 rpm for 10 minutes and the supernatant was assayed for CGTase activity and used as crude enzyme solution. The culture was centrifuged at 10, 000 rpm for 20 minutes at 4 ^{0}C to remove the cells and the cell free supernatant was recovered as the crude enzyme fraction.

Purification of the enzyme

The crude enzyme was purified in three steps including ammonium sulphate precipitation and ion exchange chromatography followed by gel permeation chromatography. Another method used for enzyme purification include starch adsorption chromatography followed by gel filtration chromatography and ultra-filtration methods (Asharaf et al., 2011).

DEAE Cellulose column chromatography and Ultrafiltration.

The crude enzyme was brought to 60% ammonium sulphate saturation. The pellet (12000xg for 20 minutes) was dissolved in 25 mM phosphate buffer (pH 7.5), and dialyzed overnight against the same buffer .The dialysate was divided into two portions: from which one was applied to DEAE –cellulose column (2.5 x 10 cm) equilibrated with 50 mM phosphate buffer (pH 7.5) and eluted with linear gradients of NaCl (0.1 to 1.0 M) at a flow rate of 0.5 ml / min. The active fractions were pooled and concentrated by ultra-filtration using a membrane with cut off value of 30 kDa molecular weight (Cao et al., 2005) for further studies.

Gel filtration chromatography

The concentrated active fractions of CGTase obtained by ion exchange chromatography followed by ultrafiltration were applied to a Sephadex G-100 column (1 cm x 20 cm) equilibrated with phosphate buffer at pH 7.5. After column washing with the same buffer, the enzyme was eluted with buffer containing 0.1 M NaCl at a flow rate of 0.7 mL /min. The active fractions were collected and was concentrated by ultrafiltration using a membrane of 30 kDa cut off. Absorbance at 280 nm and CGTase activity in protein fractions were estimated (Nomoto et al., 1986). All the purification steps were performed in cold room at 4 ^{0}C.

Starch Adsorption Method

As described previously, two methods were employed for the purification of CGTase enzyme, one is ammonium sulphate precipitation followed by ion exchange chromatography and gel filtration chromatography. Yet another method followed here is ammonium sulphate precipitation and starch adsorption chromatography.

Starch adsorption (Ferrarotti et al., 1996) was carried out as follows. The pellet obtained through 60% ammonium sulphate precipitation, after dialysis was concentrated using a concentrator. Later an ultrafilter was mixed with 5% starch solution and gently stirred to 60 minutes at 4 ^{0}C .The CGTase enzyme which absorbs on starch was collected by centrifugation at 4000g for 20 minutes at 4 ^{0}C.The pellet was washed twice with 10 mM phosphate buffer (pH 6.6). To extract the adsorbed CGTase enzyme, 1 mM solution of β–CD in 10mM phosphate buffer was added with mechanical stirring at 37 ^{0}C for 30 minutes. The supernatant was collected by centrifugation and dialyzed against 10 mM phosphate buffer at pH 6.6 at 4^{0}C .The dialyzed sample was concentrated and stored for β –CD production studies.

Enzyme assay

CGTase activity was assayed according to the phenolphthalein test described by Kaneko et al (1987). Enzyme solution (0.1 ml) was added to 1 ml of gelatinized soluble starch solution 2% (w/v) in 100mM phosphate buffer pH (6.6) and incubated at 65 ^{0}C for 20 minutes. The reaction was stopped by adding 3.5 ml of 30 mM NaOH and then 0.5 ml of phenolphthalein solution (0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3) was added to the reaction mixture and left to stand at room temperature for 15 minutes. The concentration of β –CD produced by CGTase action on starch was determined by the change in absorbance at 550 nm based on starch curve of β –CD. One unit of enzyme was defined as the amount of enzyme required for the production of 1mg of β –CD per minute from the substrate**.**

Protein Determination

Protein concentration were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard protein. For the chromatographic profile, the protein content in each fraction was measured by light absorption at 280 nm.

Polyacrylamide gel electrophoresis (PAGE)

The molecular weight of the purified enzyme was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) on a vertical slab gel using 12% poly acryl amide gel at a constant current of 25 – 30 mA using standard molecular markers ranging from 25 kDa to 150 kDa.

CGTase characterization

Effect of pH and temperature on purified CGTase enzyme

The effect of pH on the activity of the purified enzyme was measured with buffer of different pH values ranging from 4.0 to 10.0 at 65 ^{0}C for 20 minutes using 0.1M sodium acetate buffer of pH 4.0 – 6.0, 0.1 M phosphate buffer of pH 6.0- 8.0 and 0.1M glycine - NaOH buffer of pH 9.0 - 10.0 (Jemli et al., 2007). The effect of temperature on CGTase activity of the purified enzyme was studied by incubating the purified enzymes at temperatures ranging from 40^{0}C – 90^{0}C for 20 minutes in 100 mM sodium phosphate buffer of pH 6.5 (Sian et al 2005).

Effect of pH and temperature on CGTase stability

The pH stability of purified CGTase enzymes was measured by incubating 0.1ml of purified CGTase enzyme with 0.2 ml of sodium acetate buffer (0.1M, pH 4.0 – 6.0), sodium phosphate buffer (50 mM, pH 6.0 - 8.0,), 0.1 M glycine /NaOH buffer (pH 9.0 – 10.0) and carbonate/ bicarbonate buffer (pH 10.0 – 12.0) respectively at 65^{0}C, without substrate for 1 hour and 3 hours. Then the relative activity of the enzyme was assayed by standard assay method as described earlier (Kaneko at al 1987).

The temperature stability of the purified enzyme was measured by incubating the pure enzyme at temperatures from 40^{0}C - 90^{0}C for 1 hour, then the residual activity of the enzyme was assayed by the standard assay method (Jemli et al, 2007).

Effect of metal ions on enzyme activity

The effects of metal ions on the CGTase activity was investigated by incubating purified enzyme with divalent cations like 1mM Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+} in phosphate buffer (50 mM, pH 6.5), at 30 ^{0}C for 30 minutes. The residual activity percentage was measured at the optimum conditions using starch as substrate and compared with that of the control without any metal ions, taking the control as 100% positive (Cao et al 2005).

Kinectic parameters

The Km and Vmax was determined by incubating 0.05ml of purified enzyme with starch substrate (soluble, corn and potato) at different concentrations (0.5-10mg/ml) in phosphate buffer (50 mM, pH 6.5). The assay was performed according to standard enzyme assay. The kinetic constants K_m and V_{max} were estimated using Michaelis – Mention equation and double – reciprocal plot known as Line Weaver – Burk plot (Doukyu et al., 2003).

Statistical analysis

The mean values and standard errors were calculated from the data obtained from six different experiments. Analysis of variance was performed by one way ANOVA procedures followed by Tukey HSD post HOC tests using SPSS 11.5 software. Statistical differences atp < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Isolation of CGTase producing bacteria

Alkalophilic CGTase producing bacteria was isolated from soil samples obtained from parambikulam wild life sanctuary and identified as *Leucobacter* sp., a potent CGTase producer and an alkalophilic gram positive, rod shaped organism, which was deposited in the GenBank MTCC Chandigarh with accession number -10603.

Purification of CGTase enzyme

The crude enzyme was purified to homogeneity by using three step procedures including 60% ammonium sulphate precipitation, DEAE cellulose column chromatography followed by gel permeation chromatography. Another method used for purification was 60% ammonium sulphate precipitation followed by starch adsorption chromatography followed by gel permeation chromatography as the final step. The purification steps and its summary are presented in the Table.1.

The maximum CGTase enzyme activity was noticed with 60% ammonium sulphate saturation. The crude preparation for CGTase showed 189 U/mg of specific enzyme

Table 1. Summary of the purification of CGTase enzyme produced by

	Volume(ml)	Total Activity U/ml	Specific activity U/mg	Yield %	Purification Fold.
Crude Enzyme	1000ml	1, 89, 000	189	100%	1
Ammonium sulphate (60%) saturation.	25ml	1, 39, 859	5594.36	74%	29.6
DEAE cellulose chromatography.	3ml	46, 285	15, 428	25%	81.6
Starch adsorption chromatography.	20 ml	10, 400	520	55%	5.5
Sephadex G100 columchromatography.	2ml	36, 860	**18, 430**	19.5%	98

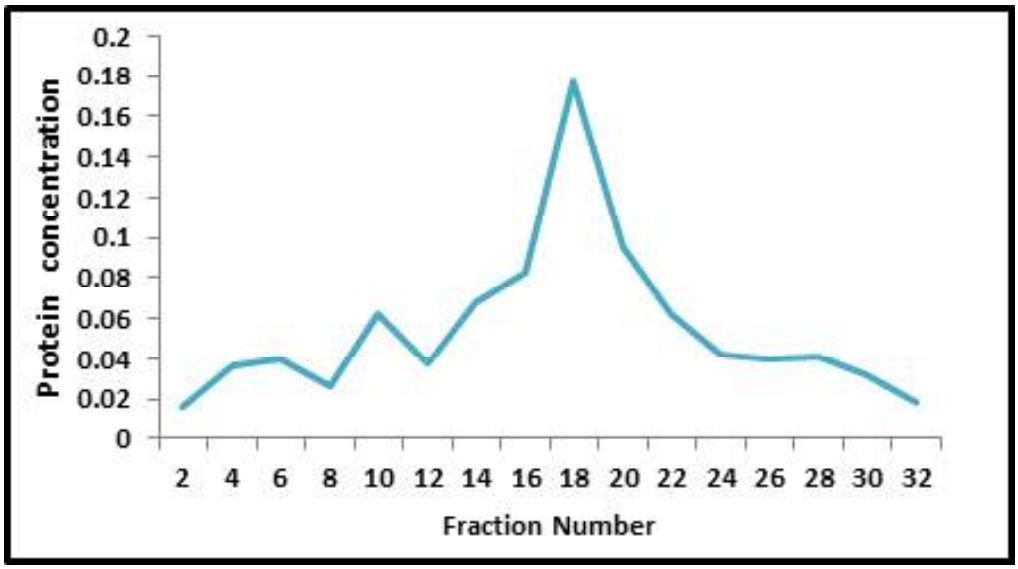

Fig. 1. Elution profile of CGTase enzyme by DEAE Cellulose column chromatography

activity, which was increased to 5595 U/mg of protein after ammonium sulphate precipitation.

Ion exchange chromatography (IEC)

Anion exchange DEAE cellulose was used for IEC. The elution profile of the anion exchange chromatography (Fig :1) showed one main peak corresponding to the purified CGTase. The maximum enzyme activity fractions were 16 – 19, which was pooled and lyophilized, and stored for further studies.

Sephadex G-100 column chromatography.

The pooled and lyophilized CGTase enzyme fraction of IEC was further purified by Gel permeation chromatography (GPC) or Gel filtration using Sephadex G – 100. The IEC concentrate was applied to the Sephadex G – 100 column equilibrated with phosphate buffer pH 8.0. The enzyme was eluted in the major peak at 0.3M NaCl gradient, with fraction numbers ranging from 16 – 20 (Fig :2), when compared with those of standard proteins.

Estimation of molecular weight.

The molecular weight of thee purified CGTase was estimated to be 64 kDa by SDS – PAGE and showed a single protein band (Fig :3) on 12.5% polyacrylamide gel. Most of the previously purified CGTases from various species had a molecular weight ranged between 60- 110 kDa, for instance CGTase from *Bacillus species* KC 201 is 65 kDa (Noriyuki

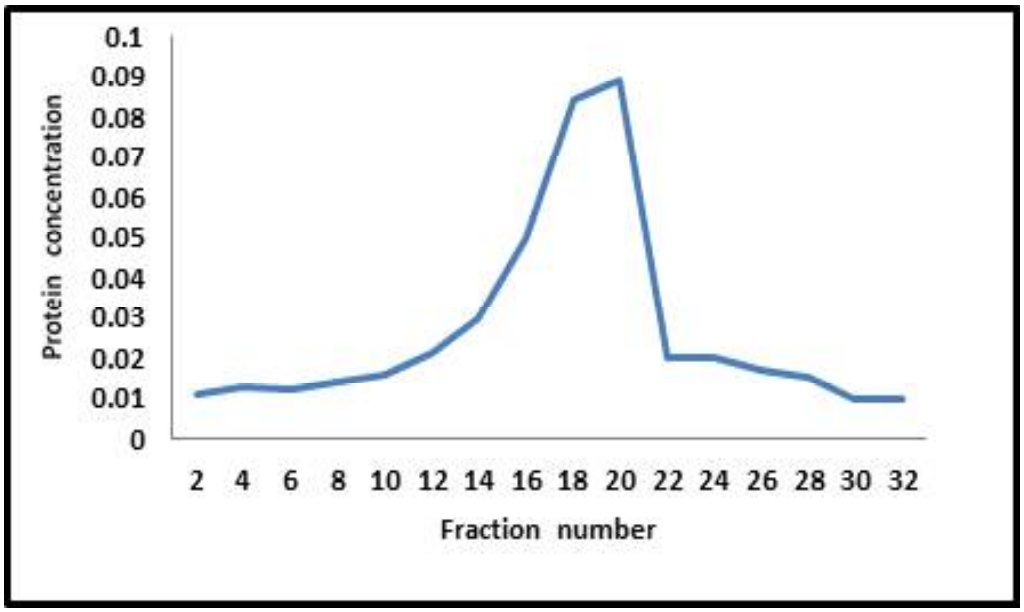

Fig. 2. Elution profile of CGTase by sephadex G-100 column chromatography

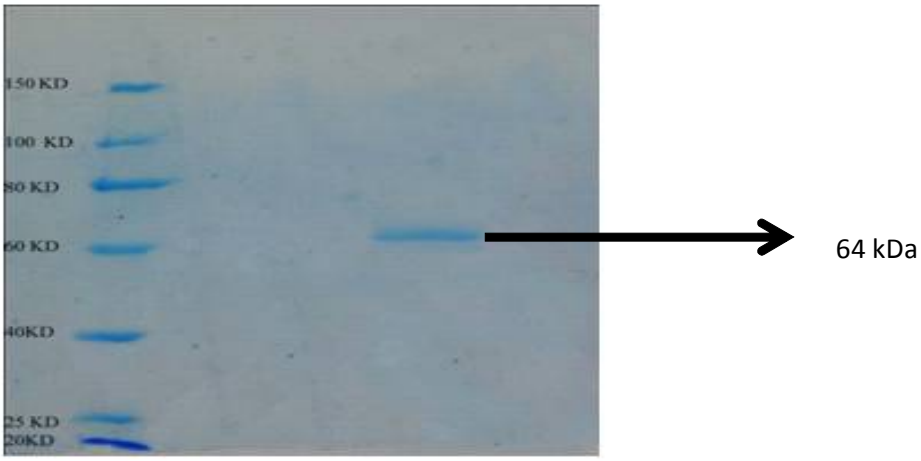

Fig. 3. SDS-PAGE analysis of purified CGTase of *Lecobacter species*

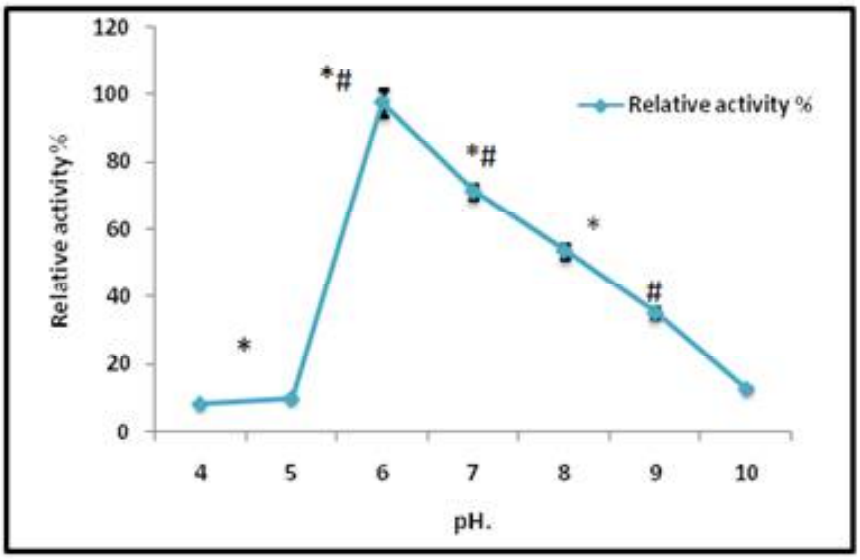

Fig. 4. Effect of pH values on purified CGTase activity. Values expressed as average of six samples ± SEM in each group. * Statistical difference compared with group I at $p<0.05$. # Statistical difference compared with group II at $p<0.05$.

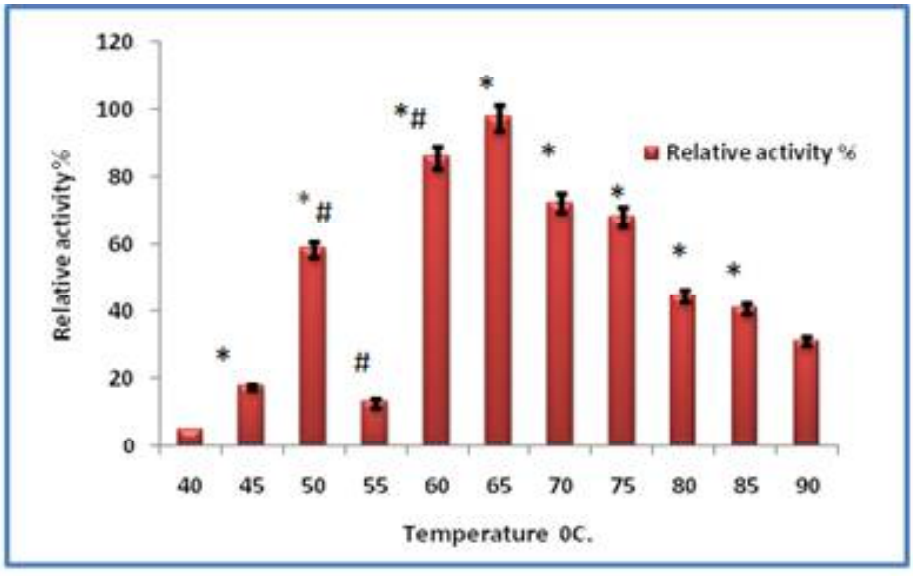

Fig. 5. Effect of temperature on purified CGTase activity. Values expressed as average of six samples ± SEM in each group. * Statistical difference compared with group I at $p<0.05$. # statistical difference compared with group II at $p<0.05$.

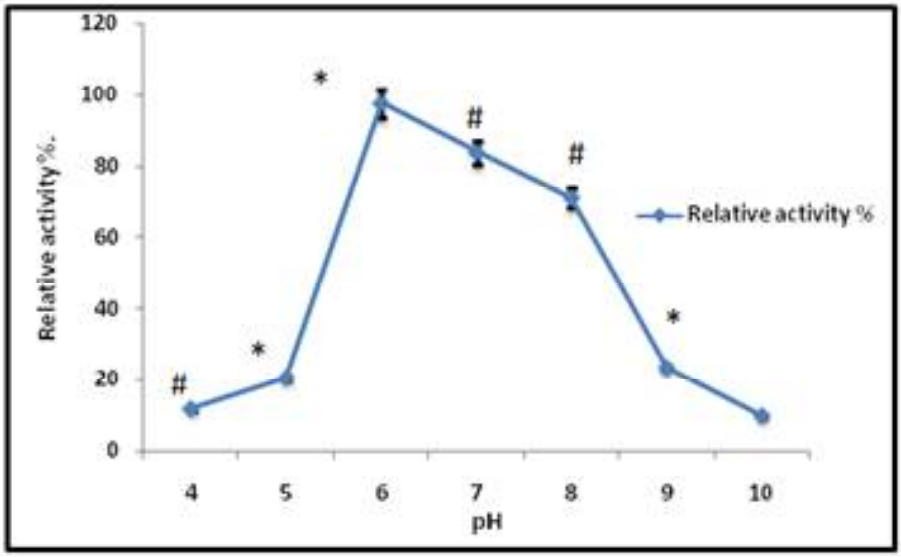

Fig. 6. pH stability on purified CGTase activity. Values expressed as average of six samples ± SEM in each group. * Statistical difference compared with group I at $p < 0.05$. # Statistical difference compared with group II at $p<0.05$.

Kitamo et al., 1992), CGTase from *Brevibacillus brevis* CD 162 is 75 kDa (Myunget et a., l 1998), CGTase from *Bacillus species* H A is 68 kDa (Nomoto et al 1968), *B. firmus* has a molecular weight of 80 KDa (Sohn et al., 1997), and *Bacillus agaradhaerens*, was 110 kDa (Martins and Hatti –Kaul, 2002).

However, CGTases with lower molecular weight have also been reported, such as 33 kDa from *Bacillus coagulans* (Sain et al., 2005), 38 kDa from *Bacillus species* (Wang et al., 1995) and as 56 kDa from *Bacillus sphaericus* strain 41 (Moriwaki et al., 2009).

CGTase characterization

Effect of pH and temperature on CGTase activity

The effect of pH and temperature on the purified CGTase activity is shown in figure: 4 and 5. The optimum pH of purified CGTase was determined as pH 6 and optimum temperature as 65 °C. At pH 7.0, 8.0 and 9.0, enzyme retains its activity by 75%, 55% and 36% respectively. The effect of temperature on CGTase activity showed that, at 70 °C, 75 °C, 80 °C and 85 °C the enzyme was active by 74%, 45% and 42% respectively.

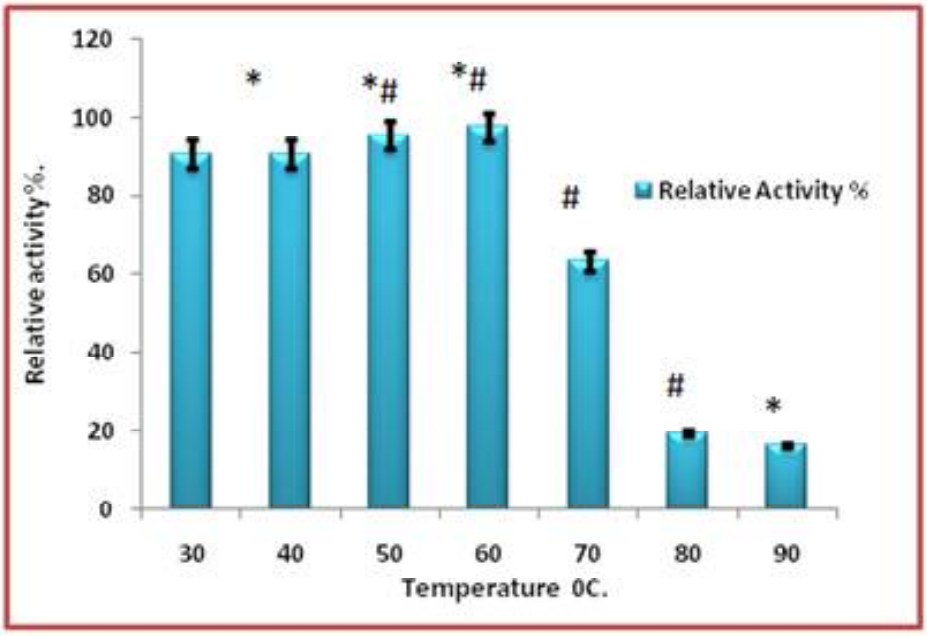

Fig. 7. Thermal stability of purified CGTase for 1 hr.

Values expressed as average of six samples ± SEM in each group. * Statistical difference compared with group I at $p<0.05$. # Statistical difference compared with group II at $p<0.05$.

Other studies, were CGTase from *Bacillus megaterium* (Pishtiyski et al., 2008) and *Alkalophilic bacillus sp G1* (Ong et al., 2008) showed 60 °C as the optimum temperature. Jung et al (2007) concluded that the optimum pH of the purified CGTase from new alkalophilic *Bacillus species BL-12* as 9.0, while Ai Noi et al (2008) found that the optimum pH of the crude CGTase from *Bacillus species* MK 6 as pH 6.0.

Effect of pH on stability of CGTase

The effect of pH on stability of CGTase is shown in figure 6 . The CGTase enzyme was stable in the pH range 6.0 – 8.0, with maximum stability at pH 6and the purified CGTase retained almost more than 86% of its initial activity between pH 6 and 7, and 73% at pH 8.0, while the pH above or below had low stability. CGTase from *Leucobacter* sp. had a narrower pH range for stability when compared to CGTase from *Bacillus megaterium* (pH 6.0 – 10.5), (Pishtiyski et al 2008), *Klebsiella pneumoniae* AS – 22 (pH 6.0 – 9.0), (Gawande and Patker 2001).

Effect of temperature on CGTase stability

The thermal stability of purified CGTase was investigated by pre-incubation of the enzyme at different temperatures (35 ^{0} C to 90 ^{0}C) at pH 6 for various time intervals before measurement of relative cyclization activities. The results were illustrated in figure 7. .

The enzyme showed thermal stability up to 70 °C for 1 hour. Maximum activity of the enzyme observed was at 65 °C. However, it began to lose about 35% of its activity around 70 °C and also lost about 79% of its activity at 90 °C. The enzyme isolated and purified had a higher temperature stability compared to CGTase from *Bacillus megaterium* (Pishtiyski et al., 2008) and *Bacillus agaradhaerens* (Martins and Hattikaul, 2002), where both showed maximum stability at 30 °C and 40 °C, respectively. Another studies showed that the thermal stability of CGTase produced from alkalophilic *Bacillus species* 277 ranged from 40 to 70 °C for 30 minutes, though the enzyme rapidly lost its activity above 70 °C (Cao et al., 2005), which is highly related to the present *Leucobacter* sp.

Effect of some metal ions on enzyme activity.

The effect of different metal ions on CGTase activity are summarized in table :2 . Ithas been observed that Ca^{2+}, Mg^{2+} and Fe^{2+} brought about 115%, 108%, 109% residual activity respectively. On the other hand, the enzyme was slightly inhibited by Mn^{2+}, Cu^{2+} and strongly inhibited by Zn^{2+}.In previous studies, the enzyme activity produced from *Bacillus megaterium* was inhibited by Zn^{2+} and Ag^{+}, but enhanced by Sr^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+} and Cu^{2+} ions (Pishtiyski et al 2008).

Table 2. Effect of some metal ions on the purified CGTase activity

Metal ions (1mM)	Residual Activity (%)
Control	100%
$CaCl_2$	115%
$ZnSO_4$	21%
$MgSO_4$	108%
$CuSO_4$	48%
$MnSO_4$	61%
$FeSO_4$	109%
EDTA	45%

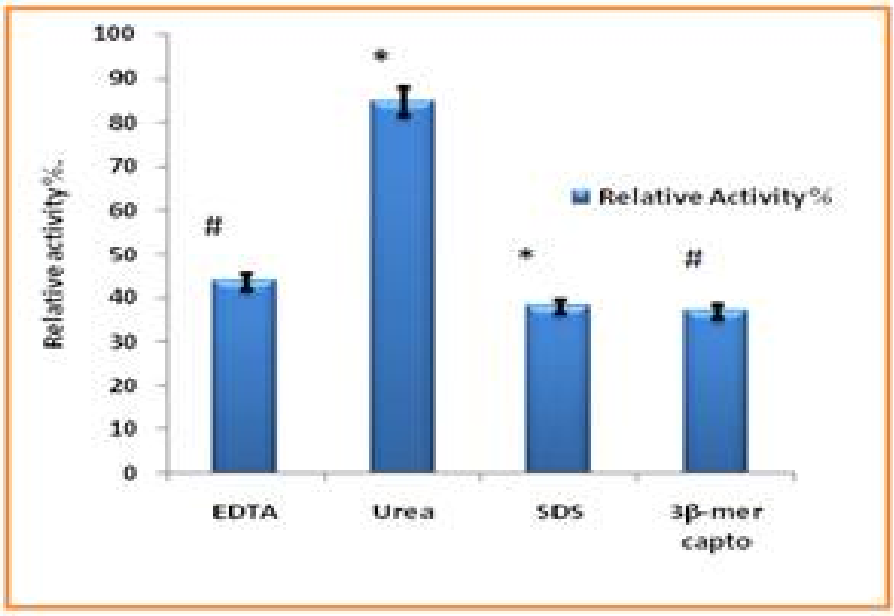

Fig. 8. Effect of enzyme inhibiters, detergents and reducing agent on CGTase activity. Values expressed as average of six samples ± SEM in each group. * Statistical difference compared with group I at $p<0.05$. # Statistical difference compared with group II at $p<0.05$.

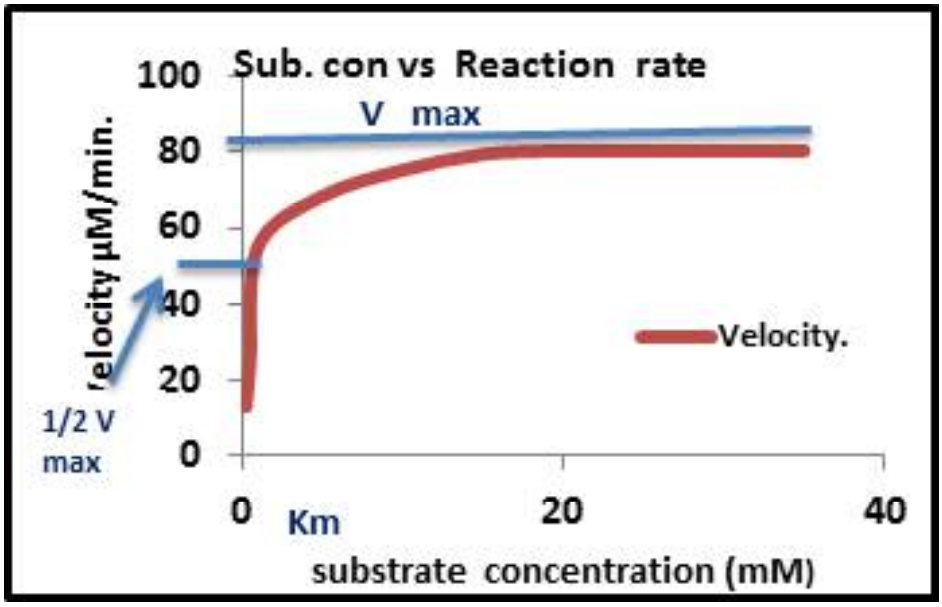

Fig. 9. CGTase shows Michaelis Menton Kinetics when acting on starch substrate.

Another study showed that the CGTase activity produced from alkalophilic *Bacillus sps* BL- 12 was inhibited by Zn2+ ions yet stimulated by $Ca^{2+,}$ which resulted in an increase upto 123 % at 1mM concentration (Jung et al., 2008).

Effect of enzyme inhibitors, detergent and reducing agent on CGTase.

EDTA partially inhibited the enzyme activity while urea showed no effect on enzyme activity. SDS and 2-β mercaptoethanol showed inhibition on enzyme activity (Figure: 8). Alkali tolerant *Bacillus species PS304* showed inhibitory effect on EDTA and β-mercapto ethanol (Prasert suntinanalert et al 1997).

Kinetic Parameters

Kinetic studies of purified CGTase were investigated by measuring initial rates of CGTase reaction at different concentrations of soluble starch ranging from 0.1 to 10 mg/ml in 100mM phosphate buffer at pH 6.6 and 65^{0}C.The kinetic constants K_m and V_{max} were estimated using Michaelis – Menton equation (Fig :9 and Fig:10) and double reciprocal plot known as Line weaver –Burk plot. The K_m and V_{max} values were 0.59µM and 81.5µM/minutes respectively. The

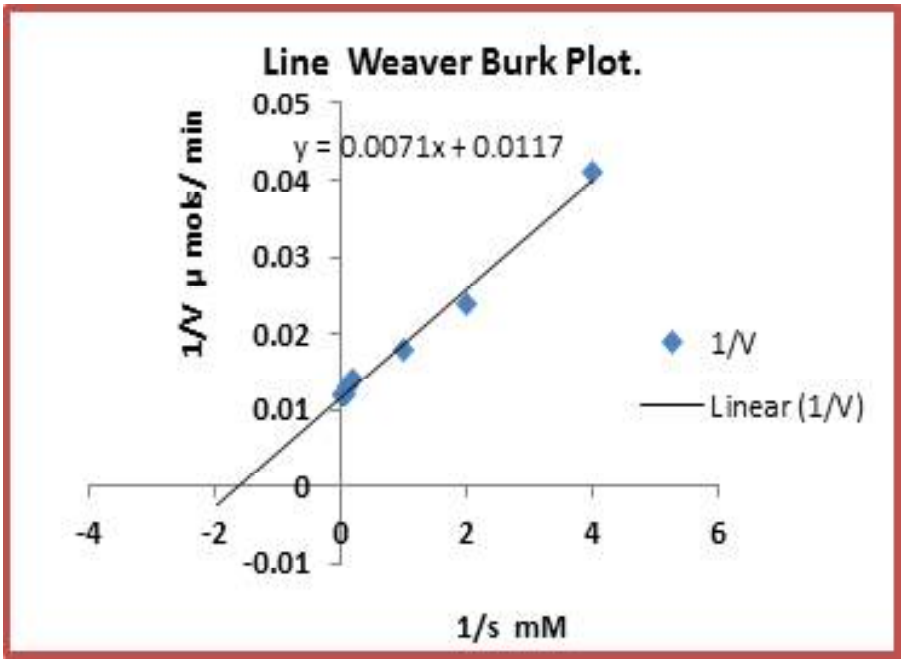

Fig. 10. Line Weaver Burk Plot for CGTase enzyme.

low value of K_m indicates the high affinity of CGTase produced by *Leucobacter* sp. towards the substrate.

Previous reports have shown that K_m values range from 0.05mM – 15.54mM (Bovetto et al., 1992, Jung et al., 2007, Nakamure et al., 1994). Different K_m values ranging from 1.77 – 5.7 mg/ml and V_{max} from 43 – 1027 U/mg have been reported by other investigators for a few *Bacillus species* CGTase (Akimaru et al., 1991, Boveto et al., 1992, Stavn and Granum 1991, Stavn and Granum 1979).

CONCLUSION

In this study, purification and characterization of CGTase from a novel alkalophilic *Leucobacter* sp. isolated from the forest area of Parambikulam Wild Life Sanctuary area is reported. Cyclodextrin glucanotransferase (CGTase) from *Leucobacter* is then effectively used for conversion of starch into cyclodextrin under a wide range of pH conditions. Enzyme purification to homogeneity was achieved by DEAE cellulose column chromatography, Ultrafiltration and gel filtration chromatography with a recovery of 19.5% activity and 98 fold purification (Purified CGTase using starch adsorption method gave an enzyme activity of 520 U/mg with 5.5 fold purification, which was utilized for the enzymatic production of β – cyclodextrin). The purified enzyme was a monomer and its molecular weight was estimated to be 64 kDa. The purification procedure was easily applicable under industrial conditions. The present enzyme can be used in the pH range of around pH 6.0 (optimum pH) to pH 9.0 and temperature range of 40 ^{0}C to 70 ^{0}C. **:** The enzyme activity was inhibited by Zn^{2+} but enhanced by $Ca^{2+,}$ Mg^{2+} and Fe^{2+} ions. The isolated *Leucobacter* can be used for β – CD production and also its relatively high affinity for starch is of interest for industrial application.

LITERATURE CITED

Abdel M, El H, Abdel A 2011. Biosynthesis of cyclodextrin glucosyltransferase by the free and immobilized cells of Bacillus cereus NRC7 in batch and continuous cultures. *J Appl Microbiol* **111**:1129–1137

Ai-Noi, S., A.A. Suraini, A. Norjahan, H. Osman and K.M.I. Abdul, 2008. Optimization of cyclodextrin glycosyltransferase production by response surface methodology approach. *J. Biotechnology,* **7**: 10-18.

Akimaru, K. 1991. Purification and properties of *Bacillus coagulans* cyclomaltodextrin glucanotransferase. *J. Ferm. Bioeng*., **71**, 322-328.

Ashraf, F. El-baz., Ahmed, A. Abd El-Rahman., and Ahmed Sobhy. 2011, Purification and some properties of cyclodextrin glucanotransferase from alkalophilic *bacillus* sp. *Annals of Agric. Sci. Moshtohor* **49** (2) 135 – 141.

Bovetto, L., Backer, D., Villette, J., Sicard, P., Bouquelet S: 1992. Cyclomaltodextrin glucanotransferase from *Bacillus circulans* E192. *Biotechnol Appl Biochem,* **15**:48–58.

Cao, X., Z . Jin, X. Wang and F . Chen, 2005 . A novel cyclodextrins glycosyltransferase from an alkalophilic *bacillis species* purification and characterization. *Food Res . Int,* **38**: 309.314.

Doukyu, N., Kuwahara, H., Ano, R., 2003. Isolation of *Paenibacillus illiniosensis* that produces cyclodextrin glucanotransferase resistant to organic solvents. *Biol. sci. Biotechnol. Biochem.* **67** (2): 334-340.

Ferrarotti, S.A., Rosso, A.M., Marechal, M.A., Krymkiewicz, N., Marechal, L.R., 1996. Isolation of two strain (S-R Type) of Bacillus circulans and purification of a cyclomaltodextrin-glucanotransferase. *Cellular and Molecular Biology*, **42** : 653-657.

Gawande, B.N., Patkar, A.Y., 2001. Purification and properties of a novel raw starch degrading-cyclodextrin glycosyltransferase from *Klebsiella pneumonia* AS- 22. Enzy. *Microb. Technol*. **28**: 735-743.

Hamoudi M, Fattal E, Gueutina C, Nicolas V, Bochota A 2011. Beads made of cyclodextrin and oil for the oral delivery of lipophilic drugs: In vitro studies in simulated gastro-intestinal fluids. Int J Pharm 416:507–514

Jemli, S., Messaoud, E.B., Ayadi-Zouari, D., Naili, B., Khemakhem, B., Bejar, S.A., 2007. Beta-cyclodextrin glycosyl transferase from a newly isolated *Paenibacillus pabuli* US132 strain: Purification, properties and potential use in bread- making. *Bio chem. Eng. J.* **34**: 44-50.

Jung, S., Kim, T., Lim, K., Lee, Y., 2007. Catalytic properties of betacyclodextrin glucanotransferase from alkalophilic *Bacillus sp. BL-12* and intermolecular transglycosylation of stevioside. *Biotechnology and Bioprocess Engineering*, 12, 207-212

Kaneko, T., Kato, T., Nakamura, N., and Horikoshi, K., 1987. Spectrophotometric Determination of Cyclization Activity of β-Cyclodextrin- Forming Cyclodextrin Glucanotransferase. *Journal Japan Society Starch Science.* **34**: 45-48.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. J.Biol.Chem 193: 265 (The original method)

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Marcon F, Mathiron D, Pilard S, Lemaire-Hurtel A, Dubaele J, Djedaini-Pilard F 2009. Development and formulation of a 0.2% oral solution of midazolam containing ã-cyclodextrin. Int J Pharm 379:244–250.

Marlene M M M, Karel H S, Reynaldo V S, Arley P R, Héctor L R1 and Juan J T-L 2012. Partial purification and properties of cyclodextrin glycosiltransferase (CGTase) from alkalophilic Bacillus species. SpringerPlus 2012, 1:61

Martins, R.F., Hatti-Kaul, R; 2002. A new cyclodextrin glycosyltransferase from an alkaliphilic *Bacillus agaradhaerens* isolate: purification and characterization. *Enzyme Microb. Technol.* **30**: 116-124.

Moriwaki, C.; Ferreira, L.R.; Rodella, J.R.T.; Matioli, G. 2009. A novel cyclodextrin glycosyltransferase from *Bacillus sphaericus* strain 41: Production, characterization and catalytic properties. *Biochem.Eng.J,* **48** : 124-131.

Myung, H.K., Cheon, B.S. and Tae, K.O. 1998. Cloning and Sequencing of a cyclodextrin glycosltransferase gene from *Brevibacillus brevis* CD 162 and its

expression in *Escherichia coli. FEMS Microbiology letters*. **164**: 411-418.

Nomoto, M., Chen, C. C. and Sheu, D. C. 1986. Purification and Characterization of Cyclodextrin Glucanotransferase from an Alkalophilic Bacterium of Taiwan. *Journal of Agricultural Biology Chemistry*. **50**(11): 2701 –2707.

Noriyuki Kitamoto, Yukio Kito, Tetsuya Kimura ., Kunio Ohmiya. 1992. Cloning and sequencing of the gene encoding cyclodextrin glucanotransferase from *Bacillus sp*. KC201 *Journal of Fermentation and Bioengineering* **74** (6) :345 – 351.

Ong R.M; Goh K.M., Mahadi, N., Hassan, O., Rahman, R.N.Z; Illias, R.M 2008. Cloning, extracellular expression and characterization of a predominant β CGTase from *Bacillus* sp. G1 in *E. coli. J. Ind Microbiol. Biotechnol*. **35**: 1705-1714.

Prasert Suntinanalert, Jintana Petmaneechote, Nisarat Damnian and Nongporn Hutadilok- Towatana 1997. Purification and properties of Cyclodextrin glycosyltransferase (CGTase) from an alkaline tolerant *Bacillus species* ps304. *J.Sci.Soc. Thailand,* **23** 381- 388.

Pishtiyski, I., Popova, V., Zhekova, B. 2008. Characterization of Cyclodextrin Glucanotransferase produced by *Bacillus megaterium. Appl Biochem Biotechnol*. **144**:263–272.

Sian, H.K., Said M, Hassan O, Kamaruddin K, Ismail AF, Rahman RA 2005. Purification and characterization of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. G1. *Proc. Biochem*. **40**: 1101-1111.

.Stavn, A. and Granum, P.E., 1979. Purification and physicochemical properties of an extra-cellular cycloamylose (cyclodextrin) glucanotransferase from *Bacillus macerans*. *Carbohyd. Res*., **75**: 243- 250.

Sohn, C. B .Kim, S.A, Park Y.A, Kim M.H, Moon S.K and Jang S .A 1997. Characterization of cyclodextrin glycosy ltransferase from *Bacillus firmus. J. Korean Soc .Food Sci Nutr,* 26(2), 351- 357.

Wang, G. S, Chen, P.L, Liu, Y.T and Wang, L.H 1995. Purification of cyclodextrin glucosyltransferase from a mutant of *Bacillus species*. Rept Taiwan Sugar Res. Inst. 150:53 66.

Wang J, Cao Y, Sun B, Wang C 2011. Physicochemical and release characterization of garlic oil- β-cyclodextrin inclusion complexes. *Food Chem*. **127**:1680–1685

Received on 16-04-2018 Accepted on 20-04-2018