Chapter 2

Screening of alkaliphilic bacteria for production of Cyclodextrin glycosyl transferase

2.1 Introduction

2.2 Materials and methods

2.2.1 Screening of alkaliphilic bacteria from Lonar Lake for production of CGTase

2.2.2 Production of CGTase in shake flask

2.2.3 Enzyme Assay

2.2.4 Tilden and Hudson Microscopic Test

2.2.5 Trichloro ethylene Test

2.2.6 Detection of Cyclodextrins by HPLC

2.3 Results

2.3.1 Screening of alkaliphilic bacteria from Lonar Lake for production of CGTase

2.3.2 Production of CGTase in shake flask culture

2.3.3 Detection of cyclodextrins by microscopic and precipitation tests

2.3.4 Detection of cyclodextrins by HPLC

2.3 Discussion

2.4 Conclusions
2.1 Introduction

Cyclodextrin glycosyl transferase (CGTase) is a microbial enzyme that generates cyclodextrin (CD) from starch. CD’s and their derivatives are used in pharmaceutical, food and cosmetic industry. Most of the CGTase producing organisms belong to the genera *Bacillus* and *Klebsiella*. The screening of microorganisms for production of CGTase is performed by conventional methods. The general approach used is primary screening on starch agar plate followed by secondary screening on phenolphthalein methyl orange medium (PMO medium). This medium was developed by Park *et al.* (1989) by incorporation of the dyes phenolphthalein and methyl orange in Horikoshi medium. The CGTase production can also be detected colorimetrically using phenolphthalein assay as described by Goel and Nene (1995).

2.1.1 Principle of phenolphthalein assay

The decolorization of phenolphthalein solution in two variants cannot be attributed to the shift in pH towards the acid range, since the suspension or solution is demonstrably alkaline (pH = 10 – 11). When the magenta phenolphthalein solution is added to β-cyclodextrin or to the β-cyclodextrin solution, the cone-shaped β-cyclodextrin forms a host-guest complex as seen in Fig. 2.1 with the guest molecule phenolphthalein, due to van der Waals interactions.

![Molecular structures of phenolphthalein at pH < 8.5 and pH > 9](image)

Since the molecular structure is non-ionic and therefore less polar in acid and neutral conditions, it can be assumed that the phenolphthalein is complexated in this form, which would also explain the decolorization of the solution. UV-VIS
spectroscopy of different substances and complexes has shown that the phenolphthalein is present in the cyclodextrin in the dianionic form. During the formation of the host-guest complex, the phenolphthalein dianion is complexated by the formation of three hydrogen bonds to the cyclodextrin molecule. Therefore the van der Waals forces between the guest molecule and the non-polar cavity of the cyclodextrin are not the significant forces in this complex.

Thus when CGTase is produced in starch containing nutrient medium, the enzyme breaks down starch to release cyclodextrin molecules. When phenolphthalein reagent is added to the reaction mixture in alkaline condition, cyclodextrin forms a complex with the pink dye and the reaction mixture becomes colorless.

![Fig. 2.2: Schematic diagram of the hydrogen bonds between the β-cyclodextrin molecule and the phenolphthalein dianion](image)

Higher the concentration of CD, lesser is the absorbance of reaction mixture. A standard curve of decrease in absorbance versus increasing standard solution of cyclodextrin (20-200 µg/ml) can be plotted and the concentration of unknown solution can be extrapolated from the standard graph.

Researchers have used phenolphthalein plate assay for screening of bacteria for production of CGTase. Noi et al. (2008) have screened bacteria from soil samples using Horikoshi medium containing phenolphthalein by plate assay. Bonilha et al. (2006) have used phenolphthalein plate assay for qualitative analysis of CGTase from B. licheniformis. Aziz et al. (2007) have developed a rapid and modified phenolphthalein plate assay for screening CGTase producing organisms from soil.
In the present studies, alkaliphilic bacteria isolated from alkaline Lonar lake, India were screened for production of CGTase using starch hydrolysis plate assay, Horikoshi medium containing phenolphthalein and methyl orange (Park et al., 1989) and phenolphthalein colorimetric assay (Goel and Nene, 1995).

2.2 Materials and Methods

2.2.1 Screening of alkaliphilic bacteria from Lonar Lake for production of CGTase

The protocol for screening of CGTase producers from Lonar lake is outlined in Fig. 2.3.

Aerobic alkaliphilic bacteria previously isolated and identified from Lonar Lake were screened for hydrolysis of starch using Nutrient agar containing 1 % starch. Organisms showing starch hydrolytic activity were further screened for CGTase production on Phenolphthalein methyl orange agar medium containing (g/L) soluble starch 10, polypeptone 5, yeast extract 5, K$_2$HPO$_4$ 1, MgSO$_4$. 7H$_2$O 0.2, Na$_2$CO$_3$ 10, agar 15, phenolphthalein 0.3 and methyl orange 0.1. The organisms showing yellow halo on phenolphthalein methyl orange agar medium were selected for production of CGTase which was confirmed by CGTase assay using phenolphthalein reagent assay (PHP assay) as described by Goel and Nene (1995).

2.2.2 Production of CGTase in shake flask

The alkaliphilic bacteria were inoculated in Nutrient medium containing (g/L) soluble potato starch 10, peptone 10, yeast extract 5 and sodium chloride 5 for production of CGTase. The pH was maintained as 10 by addition of sterile 10 % sodium carbonate after autoclaving. The organism was cultivated in 250 ml flasks containing 50 ml medium incubated in an orbital shaker at 150 rpm at 30°C for 24 hours. After 24 h, the broth was centrifuged at 10,000 g at 4°C for 20 min. The cell free supernatant was used as crude enzyme and the enzyme activity was estimated as described below.
Fig 2.3: Flowchart of screening of alkaliphilic bacteria for production of CGTase
2.2.3 Enzyme Assay

The CGTase assay was performed using phenolphthalein reagent as described by Goel and Nene (1995). 100 µl of crude enzyme extract was added to 1 ml of 1 % soluble potato starch in 0.005 M Tris Cl buffer pH 8.5 and incubated at 60°C for 20 min. After incubation, this reaction mixture was cooled in ice. 4 ml of 1mM phenolphthalein reagent was added to the tubes and the absorbance measured immediately at 550 nm. The amount of β-CD produced is estimated from standard graph of 50 – 200 µg /ml of standard β-CD (Sigma-Aldrich) against decrease in absorbance. One unit of CGTase activity is defined as an amount of enzyme required to produce 1 µg of β-CD /ml/min.

The protein content was determined using bovine serum albumin as standard by the method described by Bradford (1976).

2.2.4 Tilden and Hudson Microscopic Test

Starch substrate is prepared by adding 3 % soluble potato starch in 0.05 M Tris-HCl buffer of pH 7.2. Five hundred µl of appropriately diluted enzyme was incubated with 1 ml of soluble starch and incubated at 60°C. Three drops of reaction mixture (approx 300 µl) are withdrawn every 5 minutes and mixed with 1 drop of Iodine and observed under a light microscope under high power (Tilden and Hudson, 1939).

2.2.5 Trichloroethylene Test

The enzyme is diluted serially in 50 mM Tris HCl buffer of pH 8.5. One ml of enzyme is added to 5 ml of 2 % starch prepared in above mentioned buffer. The reaction mixture is incubated at 60°C. After 48 h, 2.5 ml of Trichloroethylene (TCE) is added to the reaction mixture. The contents are mixed vigorously and kept overnight. White precipitate of CD-TCE complex confirms the presence of CGTase. Maximum enzyme dilution which gave distinct precipitation was defined as enzyme activity (Nomoto et al., 1986).
2.2.6 Detection of Cyclodextrins by HPLC

The amount of β- Cyclodextrin produced by the CGTase enzyme in shake flask fermentation was estimated by HPLC. Perkin Elmer Amino column (5 µm, 250 X 4.6) was used. Mobile phase was acetonitrile: water (65:35) and detection was by online Perkin Elmer series-200 Refractive Index detector. The reaction mixture consisted of 100 microliters of enzyme and 1 ml of 1% starch. The mixture was incubated at 60°C for 20 min. 0.5 ml of this reaction mixture was mixed with equal volumes of HPLC grade Acetonitrile (Merck) and centrifuged at 20,000 g for 15 min at room temperature and then filtered through a 0.45 µ filter (Millipore, USA). 10 µl was injected in the column with a syringe and the pressure was maintained between 2800 to 3070 psi. The solutions of standard cyclodextrins were prepared in HPLC grade water. The output was read using a Total Chrom Navigator Software.

2.3 Results

2.3.1 Screening of alkaliphilic bacteria for CGTase producers

15 representative out of total 40 alkaliphilic starch hydrolyzing isolates from Lonar lake were screened for starch hydrolytic activity and CGTase activity. Six isolates were found positive for CGTase production on phenolphthalein methyl orange medium. The results of confirmation of CGTase production by phenolphthalein colorimetric assay are presented in Table 2.1.
Table 2.1: Screening of alkaliphilic bacteria from Lonar lake for production of CGTase

<table>
<thead>
<tr>
<th>No.</th>
<th>MCM number</th>
<th>Name of organism</th>
<th>Starch Hydrolysis (Amylase)</th>
<th>PHP Assay (CGTase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MCM B-1001</td>
<td><em>Bacillus cereus</em></td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>MCM B-1016</td>
<td><em>B. firmus</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>MCM B-1036</td>
<td><em>B. flexus</em></td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>4</td>
<td>MCM B-1044</td>
<td><em>B. fusiformis</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>MCM B-1010</td>
<td><em>B. licheniformis</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>MCM B-1035</td>
<td><em>B. benzoivorans</em></td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>7</td>
<td>MCM B-1038</td>
<td><em>B. cohnii</em></td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>8</td>
<td>MCM B-1041</td>
<td><em>Alkalibacillus haloalkaliphilus</em></td>
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<td>--</td>
</tr>
<tr>
<td>9</td>
<td>MCM B-1034</td>
<td><em>Paenibacillus sp L55</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>MCM B-1018</td>
<td><em>Vagococcus carniphilus</em></td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>11</td>
<td>MCM B-1027</td>
<td><em>Halomonas campisalis</em></td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>12</td>
<td>MCM B-1025</td>
<td><em>Lake Bogoria isolate 25 B1</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>MCM B-1046</td>
<td><em>Alkalimonas delamerensis</em></td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>14</td>
<td>MCM B-1021</td>
<td><em>Exiguobacterium aurantiacum</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>MCM B-1006</td>
<td><em>Arthrobacter mysorens</em></td>
<td>+</td>
<td>--</td>
</tr>
</tbody>
</table>

The six isolates showing positive CGTase activity are *Bacillus firmus, Bacillus fusiformis, Bacillus licheniformis, Paenibacillus sp L55, Exiguobacterium aurantiacum* and *Lake Bogoria isolate 25 B1*. To the best of our knowledge this is the first report of alkaliphilic *Exiguobacterium aurantiacum* and *Lake Bogoria isolate 25 B1* for CGTase production.
2.3.2 Production of CGTase in shake flask culture

CGTase was produced in 250 ml flask under shake culture condition. After 24 h, the broth was centrifuged and assessed for production of CGTase by colorimetric phenolphthalein assay (Fig. 2.4) showing disappearance of pink colour.

Fig. 2.4: Colorimetric phenolphthalein assay showing disappearance of pink colour in test

The cyclodextrin produced by CGTase forms a complex with the phenolphthalein leading to disappearance of pink colour of phenolphthalein in alkaline condition. The results of estimation of β-cyclodextrin produced and specific activity of the CGTase are presented in Fig. 2.5.

Fig. 2.5: Amount of β-CD produced and specific activity of Lonar lake isolates
It was observed that *Exiguobacterium aurantiacum* produced maximum β-cyclodextrin (150µg/ml) followed by *Bacillus licheniformis* and *Paenibacillus* sp. L55 (Fig. 2.5). *B. licheniformis* isolated from cassava culture soil has been reported by Bonilha *et al.* (2006). Higuti *et al.* (2004) have reported production of CGTase from *Bacillus firmus*. However there is no report on production of CGTase from *Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1 for CGTase production.

### 2.3.3 Detection of cyclodextrins by microscopic and precipitation tests

Tilden and Hudson test is one of the oldest and classical methods of measuring CGTase enzyme activity. The results of the test are observed microscopically and presented in Fig. 2.6.

![Fig. 2.6](image)

**Fig. 2.6 :** Detection of CD using microscopic assay. A) Starch granules (Control), B) Starch with CGTase (0 min), C) Colorless starch granules with CGTase (10 min) and D) Starch granules with CGTase showing typical dichoric crystals (30 min).

For Tilden and Hudson test, 3 % starch was incubated with crude cell free supernatant of *B. licheniformis, Paenibacillus* sp. L55 and *E. aurantiacum*. This was a time bound test and the end point was conversion of blue colored starch
granules to colorless in 30 min. The end point of CGTase activity for \textit{Paenibacillus} sp. L55 and \textit{B. licheniformis} was observed by addition of concentrated enzyme (2-3 U/ml) while end point of CGTase from \textit{E. aurantiacum} was observed by addition of 1:2 diluted crude enzyme with activity of 1.5 U/ml. Typical hexagonal and dichoric crystals of starch-iodine complex were observed in reaction mixture containing CGTase from \textit{E. aurantiacum} as described by Szejtli (1988).

The Trichlororethylene (TCE) test was used to observe typical precipitation pattern. TCE assay is used to qualitatively determine the concentration of CGTase in two fold which produces CD that form TCE-CD complex precipitate. The enzyme activity of crude CGTase of all the six isolates varied from $2^2$ to $2^{10}$ dilutions for all strains which are similar to results obtained by Higuti \textit{et al.} (2004).

### 2.3.4 Detection of cyclodextrins by HPLC

CGTase activity can be confirmed by isolation and identification of the endproducts, i.e. cyclodextrins. Cyclodextrins were detected by HPLC. Standard $\alpha$ -CD (Fluka) showed a distinct peak at 2.8 min. Standard $\beta$ cyclodextrin showed a peak at 3.1 min. The mixture of standards showed distinct peak of $\beta$ and $\gamma$ CD at 3.1 and 5 min respectively (Fig. 2.7, a-c).

![HPLC analysis showing standard $\alpha$ cyclodextrin at retention time 2.8 min.](image.png)
b) HPLC analysis showing Standard β cyclodextrin at retention time 3.1 min.

c) HPLC analysis of mixture of β and γ cyclodextrin showing clear separation at retention time 3.1 min and 5 min respectively.
d) \(\beta\)-CD produced by *E. aurantiacum*

e) Mixture of CD’s produced by *B. licheniformis*

f) Mixture of CD’s produced by *Paenibacillus sp.L55*
g) Mixture of CD's produced by Lake Bogoria isolate 25 B

**Fig. 2.7:** Detection of CD by HPLC

HPLC analysis of CD produced by *E.aurantiacum, B.licheniformis, Paenibacillus* sp.L 55 and Lake Bogoria isolate 25 B is presented in (Fig. 2.7, d-g). All the six isolates produced β-CD which was detected at 3.1 min (Table 2.2).

**Table 2.2:** Estimation of β-cyclodextrin by HPLC

<table>
<thead>
<tr>
<th>S.No</th>
<th>Organism</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Bacillus firmus</em></td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td><em>Bacillus fusiformis</em></td>
<td>3.160</td>
</tr>
<tr>
<td>3</td>
<td><em>Bacillus licheniformis</em></td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td><em>Paenibacillus sp L55</em></td>
<td>3.179</td>
</tr>
<tr>
<td>5</td>
<td><em>Exigobacterium auranticum</em></td>
<td>3.13</td>
</tr>
<tr>
<td>6</td>
<td><em>Lake Bogoria isolate</em></td>
<td>3.18</td>
</tr>
</tbody>
</table>
*E. aurantiacum* showed single peak of β-CD while lake Bogoria isolate showed many peaks of α and γ CD with probable peaks of G1, G2 and G3 in addition to β-CD. The baseline of the samples showed many peaks as the culture filtrates were used, which may contain other oligosaccharides and traces of small peptides and sugars. Nene and Goel (1995) have also reported similar chromatographic peaks for α, β and γ CD.

### 2.4 Discussion

Rapid screening method like phenolphthalein plate assay helps in detection of microorganisms producing CGTase. Incorporation of phenolphthalein in Horikoshi medium was used by Park *et al.* (1989) for screening of CGTase producers.

Most of the producers of CGTase belong to genera *Bacillus*, *Klebsiella*, *Paenibacillus* and *Thermoanaerobacterium* (Wind *et al.*, 1995) and researchers are exploring extremophiles from various habitat for CGTase producers. Alkaliphilic bacteria isolated from alkaline soda lake of Lonar, India were assessed for CGTase production. Six isolates *Bacillus firmus*, *Bacillus fusiformis*, *Bacillus licheniformis*, *Paenibacillus sp L55*, *Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1 were found to produce CGTase. Horikoshi (1999) have reported for the first time the alkaliphilic *Bacillus* sp. 38-2 for production of CGTase. Bonilha *et al.* (2006) have reported alkaliphilic *Bacillus licheniformis* for production of CGTase.

To the best of our knowledge, this is the first report of production of CGTase from alkaliphilic Lake Bogoria isolate 25 B, *Exiguobacterium aurantiacum* and *Paenibacillus* sp L55 from soda lake of Lonar, India. Lake Bogoria isolate demonstrated very low production of CGTase with lower specific activity. The specific activity of *B. fusiformis* is highest followed by *B. licheniformis* and *Paenibacillus* sp. L55. CGTase production has been previously reported from organisms like *Paenibacillus azotofixans*, *Paenibacillus macerans* and *Paenibacillus* sp RB01 (Zhou *et al.*, 2012, Zheng *et al.*, 2011, Yenpetch *et al.*, 2011).

Generally bacteria produce a mixture of α, β and γ CD’s. *Thermoanaerobacterium thermosulfurigenes* EM1 (Wind *et al.*, 1995) and *Bacillus stearothermophilus* (Fujiwara *et al.*, 1992) are known to be α-CGTase producers, while *Bacillus*
circulans strain 251 (Lawson et al., 1994), Bacillus ohbensis (Sin et al., 1991) and Bacillus sp. 1011 (Kimura et al., 1987) are known to be β-CGTase producers. Lake Bogoria isolate and B. licheniformis produces a mixture of CD’s which is not suitable for industrial production. Bacillus sp. are known to produce a mixture of amylolytic enzymes with CGTase which makes the purification of the enzyme difficult. Paenibacillus sp. L55 and E. aurantiacum produce high amount of cyclodextrins and both these bacteria have not been reported for CGTase production. Paenibacillus sp. L55 and E. aurantiacum can thus be considered for further studies on production of CGTase.

2.5 Conclusions

Phenolphthalein plate and colorimetric assay were found to be efficient methods for screening of microorganisms for production of CGTase. Six isolates namely Bacillus firmus, Bacillus fusiformis, Bacillus licheniformis, Paenibacillus sp L55, Exiguobacterium aurantiacum and Lake Bogoria isolate 25 B1 from Lonar lake were found to produce CGTase using phenolphthalein assay. Two isolates, E. aurantiacum and Paenibacillus sp L55 demonstrated promising results in terms of enzyme activity, specific activity and CD production and were selected for further studies. To the best of our knowledge this is the first report of alkaliphilic Exiguobacterium aurantiacum and Lake Bogoria isolate 25 B1 for CGTase production and also the first report of CGTase producing Paenibacillus sp L55 from Lonar lake, India.