This chapter describes the materials and methods used in the present investigation.

**Chemicals**

All the chemicals and medium constituents used in this study were of analytical grade.

**Chemicals**

All media constituents used in this study were procured from Hi-Media, Mumbai. All other chemicals used were of analytical grade.

**Sample collection**

For isolation of active producers of cyclodextrin glycosyltransferase, the following samples were collected from various places located around coastal area of Andhra Pradesh, India and brought to laboratory; the samples were stored at 4°C till further use.

- **Sample RM** The sample was collected from rice mill waste at Rajamundry, which was brown in colour.
- **Sample SM** The sample was collected from sugar mill waste at Samalkot, which was oil having moderate organic matter and red in colour.
- **Sample FP** The sample was collected from fruit processing industry waste at Aswaraopet, where the soil was black in colour and rich in organic matter.
- **Sample JI** The sample was collected from jaggery industries wastes at Anakapally, which was red in colour and sandy soil material.
- **Sample PI** The sample was collected from paper industry waste from Aswaraopet, which was brown in colour.
- **Sample OI** The sample was collected from oil industry waste at Jangareddy gudem, where spoiled ghee and butter were dumped, which was oily and rich in proteinaceous material.
All the samples were collected in the sterile screw capped tubes and care was taken to see that the points of collection had a widely varying characteristic as possible with regard to the organic matter, moisture content, particle size, colour of soil and geographical distribution.

**Screening and Isolation of Bacteria**

Bacteria were isolated from the soil samples collected. Soil samples were suspended in normal saline, serially diluted and then plated on a Horikoshi 11 agar plate containing (w/v) 1.0% soluble starch, 0.5% is yeast extract, 0.5% is peptone, 0.1% is KH₂PO₄, 0.02% MgSO₄. 7H₂O, 0.02% phenolphathalein, 1.0% Na₂CO₃ and 1.5% agar PH7.5 (Illias *et al.*, 2002). Plates were incubated at 37°C for 24h. The bacterial colony that produced the largest clear and highest CG Tase activity were selected.

In the preliminary screening of organisms for CG Tase production by the above procedure, 25 colonies that produced large clear halo zones were selected for further studies.

These 25 isolates, having promising halo zones were transferred to a basal medium (Gawande ans Patkar, 2001) whose composition (g/L) was as follows:

- Soluble starch 10
- Yeast extract 05
- Peptone 05
- Na₂HPO₄ 01
- MgSO₄ 0.2
- Distilled water up to 1L
- pH 7.0

The inoculated medium was incubated at 30°C (250rpm) for 24h. After the incubation period these were tested for CGTase production.

**Assay of CGTase**

Assay of CGTase was carried out according to the method of Kaneko *et al.*, 1987. The method is described under Analytical Methods.
The amount of β-cyclodextrin produced was estimated from the standard graph of 0-500μg/mL β-CD concentration against absorbance. One unit of CGTase was defined as the amount of enzyme required to produce 1μmol of β-CD/min.

All the experiments were conducted in triplicate and the mean values were calculated. One of the isolates which showed significant CGTase activity was designated as TPR71H and selected for further studies.

**Characterization of TPR71H**

The morphological, cultural and biochemical characteristics of TPR71H were investigated by using various media and biochemical reactions as recommended by Bergey’s Manual of Determinative Bacteriology (1957, 1974), Bergey’s Manual of Systematic Bacteriology (1992), Mackie and McCartney Practical Medical Microbiology (Collee *et al.*, 1969) and Laboratory Manual of Fundamental Principles of Bacteriology (Salle, 1948). Uninoculated controls were always run for all the tests.
Chapter III                                                                                         Materials and Methods

MICROMORPHOLOGY

Shape of the cells

For the microscopic study of cells, the cells were grown on nutrient broth. A loopful of 24h culture was spread on the clean glass slide and observed under microscope.

Endospores

To study the presence of spores, a 72h culture was used. The experimental protocol as described by Salle 1948 was followed.

Motility

To determine the motility of cells, the hanging drop method was used employing 18h culture.

Gram’s staining

To study the Gram’s reaction of the culture, a 24h culture was used. The experimental protocol as described by Salle 1948 was followed.

CULTURAL CHARACTERISTICS

Sodium azide medium (Cruickshank, 1968)

The culture was inoculated into a tube containing sodium azide medium and incubated at 37°C for 48h. The growth pattern was recorded.

Crystal violet blood agar medium (Collee et al., 1969)

The culture was inoculated into a tube containing crystal violet blood agar medium and incubated at 37°C for 48h and the growth pattern was recorded.

Litmus milk reduction test (Mounica, 2000)

Litmus milk medium was used for this test. It is a selective medium for Enterococci. The medium was distributed in 5mL quantities into test tubes and sterilized by autoclave. The tubes were inoculated with the test organisms and incubated at 37°C.
for 4h. A colour change from mauve to white or pale yellow was considered as positive test. If there is no change in colour it was considered to be negative. The tubes were compared with control.

**Nutrient agar medium**

The culture was inoculated into a tube containing nutrient agar medium and incubated at 37°C for 48h and the growth pattern was recorded.

**Nutrient broth**

The culture was inoculated into a tube containing nutrient broth and incubated at 37°C for 48h. The growth pattern was recorded.

**Growth temperature range**

The organisms were inoculated into nutrient broth and incubated at the temperature range of 4 to 42°C for 7 days and the presence or absence of growth was recorded.

**Growth at different pH**

The organisms were inoculated into nutrient broth and incubated at 35°C in the pH range of 5-11 for 3 days and the presence or absence of growth was recorded.

**Heat tolerance**

The broth culture was heated in water bath at 60°C for 30 min. Then this was inoculated into nutrient broth, incubated at 37°C for 24h and observed for the presence or absence of growth.

**Growth on Sodium chloride**

For the determination of sodium chloride tolerance, 2-12 % NaCl was incorporated into the nutrient broth. The isolate was inoculated into the medium and incubated at 37°C for 3 days. The presence or absence of growth was noted.
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Materials and Methods

**BIOCHEMICAL TESTS**

The following biochemical tests were carried out by employing the prescribed media for the taxonomic studies of the isolate. Detailed composition of all the media employed in this work are given in Appendix.

**Methyl red test**

Methyl red test was performed to detect the production of acid in the fermented medium. Accumulation of acid decreases medium pH value below 4.5 which is tested with methyl red acid/base indicator. Presence of acidic pH is indicated by red colour with methyl red and taken as methyl red positive. The medium used is glucose phosphate peptone water tubes.

**Voges Proskauer test**

This test is used to identify the organism for fermentation of glucose to form acetyl methyl carbinol in buffered medium which does not change pH. Presence of this product is tested by oxidizing it to diacetyl with the addition of oxidizing agent (KOH) and shaking in air. Diacetyl reacts with alpha naphthol and gives pink colour which turns crimson later. The medium used contains glucose phosphate peptone water tubes and 40% KOH solution and 5% alpha naphthol in absolute alcohol.

**Indole test**

Indole test is used to identify the metabolic formation/accumulation of indole by test bacterial culture. This is used to identify proteolytic organisms. Indole test was performed for its presence, if present that test is given as indole positive.

**Oxidase test**

Certain bacteria possess oxidases that catalyse the transport of electrons between electron donors in bacteria and a redox dye, tetra methyl-p-phenylene diamine. The dye is reduced to a deep purple colour due to oxidase enzymes.
Materials and Methods

**Citrate Utilization Test**

Citrate utilization test was performed to determine the ability of isolate to utilize citrate as sole carbon source using Simmon’s citrate agar medium. In this medium sodium citrate was used as carbon source and ammonium ions as nitrogen source along with bromothymol blue as indicator. Citrate utilization results were analyzed by observing the change of colour of the medium from green to blue against control.

**Urease Test**

Urea utilization ability of isolated TPR71H strain was analyzed by growing the isolated in 1% urea supplemented YPD medium. The resulting rise in the pH of the medium due to the hydrolysis of urea by urease and subsequent release of ammonia was detected by the change in colour of the indicator phenol red to dark pink.

**Gelatin liquefaction (hydrolysis)**

Gelatin agar medium was used for this test (Booth, 1971). Gelatin agar was distributed into petridishes (20mL each) and allowed to solidify. The test organism was streaked at the center of the plate and incubated in an inverted position at 37°C for 3 days. The plates were flooded with mercuric chloride reagent.

The positive test indicated by causing opacity of the medium, with clear zone around gelatin-hydrolysed colony. The extent of the hydrolysis was noted by comparing the width of the clear zone around the growth.

**Starch hydrolysis (amylolytic activity) (Salle, 1948)**

Starch agar medium was used for this test. For the evaluation of amylolytic activity, the selected isolate was grown on starch agar plates for 3 days at 37°C and then flooded with weak iodine solution. The width of hydrolyzed zone around the growth and width of growth were measured.
Hydrogen sulfide test

Peptone iron agar medium was used for this test. The peptone iron agar slants were inoculated with 24h grown cultures and incubated at 37°C for 2-4 days. The slants were observed daily for any darkening along the line of growth. They were compared with uninoculated control.

Presence of the characteristic greenish-brown, brown, bluish-black or black colour of the substrate along the line of growth is indicative of H₂S production (positive test) and absence of colour indicates negative reaction.

Catalase test (Mounica, 2000)

This test is used to differentiate those bacteria that produce the enzyme catalase from non-catalase producing bacteria. Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. Several colonies of the test organism were taken with the help of sterile glass rod and transferred into a tube containing 3mL of hydrogen peroxide solution (3%). Positive test indicated by active bubbling while in negative test no bubbles formation was observed.

Nitrate reduction test

Nitrate broth medium was used for this test. The nitrate broth tube was inoculated with 24 h grown culture and incubated at 37°C for 96h. At the end of the incubation, 1 mL of clear broth was taken into a clean tube, two drops of sulphanilic acid was added followed by 2 drops of α-naphthylamine solution. The tubes were mixed well and observed for colour change. It was compared with uninoculated control. The red colour indicates positive reaction (nitrate was reduced by nitrate reductase or nitratase).
16S rRNA GENE SEQUENCING

Isolation of DNA

The chromosomal DNA of isolated strain TPR71H was isolated according to Sharma and Singh, 2005. Four mL of microbial culture was taken and the cells were pelleted in Eppendorf tubes (2.0mL) by centrifugation at 5000rpm for 5min at 37°C. The cell pellet was resuspended in 200μl of TE [10mM Tris–HCl (pH 8.0), 1mM EDTA (pH 8.0)]. 50ng of RNase was added to digest the contaminating RNA. 400 μl of Solution I [(1%, w/v-1) Sarkosyl, 0.5M NaCl, (1%, w/v) SDS] was added. The tubes were mixed properly and kept for 10min at 37°C with intermittent shaking for every 5min. Equal volume of PCI (phenol:chloroform:isoamylalcohol; 25:24:1) mixture was added immediately by inversion. The solution was centrifuged at 10,000rpm for 5 min at 37°C the supernatant was carefully transferred into a new Eppendorf tube. 0.1 volume of sodium acetate (3M, pH 5.2) and 0.6 volume of isopropanol were added and the contents were gently mixed by inverting. The DNA was precipitated in the pellet by centrifugation of Eppendorf tubes for 5min at 10,000rpm at 37°C. Pellet was washed with 1mL of 70 % ethanol and centrifuged at 10,000rpm for 3min at 37°C and the supernatant was removed and the pellet was air-dried. DNA was resuspended in 100μl of sterile deionized water and the suspension was stored at –20°C for further use.

Amplification and Sequencing of 16S rRNA Gene

In a typical procedure, 16S rRNA gene of the isolated strain, TPR71H, was amplified by PCR using 25ng of TPR71H strain chromosomal DNA, 25pmol primers [27f (5’-AGAGTTTGATCTGCTGCAG-3’) and 1500r (5’-AGAAAGGAGGATCCAGGC-3’) corresponding to the *Escherichia coli* numbering system] according to Brosius *et al*., 1978. PCR amplification was initiated by incubating at cycling parameters included denaturation at 94°C for 3min; followed by 30 cycles of denaturation at 94°C for
Materials and Methods

30 sec, annealing at 58°C for 30 sec and amplification at 72°C for 1 min and final extension at 72°C for 5 min using GeneAmp PCR system (Applied Biosystems). The amplicon was purified using Qiaquick PCR purification kit (Qiagen) and subsequently cloned into pGEM-Teasy vector system II (Promega, USA) in accordance with the manufacturer’s instruction. Plasmid from the resulting clones, extracted by using the ‘alkaline lysis method’ (Sambrook and Russell, 2001) and the same was checked for the presence of 1.5 kb inserts from Eco RI digestions prior to sequencing. Insert of recombinant plasmid p16S12 (1.5 kb 16S rRNA amplicon in pGEM T) was amplified using T7 and SP6 primers and purified. Sequencing of 1.5 kb insert of plasmid p16S12 was carried out with an ABI DNA sequeneter model 377a (Applied Biosystems) using Big-Dye Terminator kit (Applied Biosystems) using primers T7 (5' TAATACGACTCACTATAGG 3'), SP6 (5' ATTTAGGTGACACTATAG 3') and other universal 16S rRNA gene primers such as 518F (5' CAGCAGCCGCGGTAA 3'), 908F (5' AAACTCAAAGGAATTGACGG 3'), 1073R (5' ACGAGCTGACGACAGCCATG 3'), 918R (5' CCGTCAATTCCTTTGAGTTT 3') and 361R (5' CCCACTGCTGCCTCCCGTAG 3').

Phylogenetic Analysis of the Strain

The 16S rRNA gene sequence of the strain TPR71H was used as a query to search for homologous sequence in the nucleotide sequence databases by running BLASTN program (Altschul et al., 1997). The high scoring similar to 16S rRNA gene sequences were identified from the BLASTN result and retrieved from GenBank database. The identified sequences were aligned using CLUSTAL-W software (Thompson et al., 1997). Distances among selected sequences were calculated according to methods of Kimura two parameter models (Kimura, 1980). Phylogenetic trees were inferred by using the neighbor-joining (Saitou and Nei, 2004). Bootstrap analysis with the help of MEGA 3.0 software package (Kumar et al., 2004) based on 1000 resamplings.
SELECTION OF OPTIMAL PRODUCTION MEDIUM FOR CGTASE

PRODUCTION

Selection of production medium

The isolated TPR71H strain was employed in the present study to determine the most suitable production medium for the fermentative production of CGTase by submerged fermentation process.

For this purpose five different production media reported in the literature were chosen.

Composition of the media used is as follows:

**Production Medium I** (Gawande and Patkar, 20001)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>1.0%</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5%</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.5%</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.1%</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.02%</td>
</tr>
<tr>
<td>Distilled water up to</td>
<td>100mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

**Production Medium II** (John and Leon, 1968)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>2.0%</td>
</tr>
<tr>
<td>Casein hydrolyzate</td>
<td>0.5%</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5%</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.5%</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.2%</td>
</tr>
<tr>
<td>Water up to</td>
<td>100mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
</tr>
</tbody>
</table>
**Production Medium III** (Burhan *et al.*, 2005)

Soluble starch 2.0%
Yeast extract 0.5%
Peptone 0.5%
MgSO₄ 7H₂O 0.02%
K₂HPO₄ 0.1%
Water up to 100mL
pH 8.5

**Production Medium IV** (Georganta *et al.*, 1993)

Soluble starch 1.0%
Peptone 0.5%
Yeast extract 0.5%
KH₂PO₄ 0.1%
MgSO₄ 7H₂O 0.02%
Agar 3.0%
Phenolphthalein 0.03%
Sodium carbonate 1.0%
Water up to 100mL
pH 10

**Production Medium V** (Piamsook and Mitsuo, 1978)

Beef extract 0.5%
Polypeptone 1.0%
Sodium chloride 0.2%
Yeast extract 0.2%
Soluble starch 1.0%
Water up to 100mL
pH 7.2
Preparation of Inoculum

For the preparation of inoculum, the growth contents of two days old slant culture was suspended into 5mL of sterile distilled water and transferred into 45mL of inoculum medium contained in 250mL ErlenMeyer flask. The flask was incubated on the rotary shaker (220rpm) at 30°C for 24h. This suspension is used as an inoculum.

Preparation of Production medium

Five types of production media were prepared and sterilized by moist heat method.

Procedure

A 10% (5mL) of inoculum was transferred aseptically to 45mL of production medium. The flasks were kept on the rotary shaker (220rpm) at 30°C. The samples (5mL) were withdrawn for every 12h up to 72h. The samples withdrawn were centrifuged at 3000rpm for 10min and the clear supernatant was used for enzyme estimation.

ANALYTICAL METHODS

Assay of CGTase

The CGTase activity was assayed according to the method of Kaneko et al., 1987. The reaction mixture containing 1mL of 40mg of soluble starch in 0.1M potassium phosphate buffer (pH 6.0) and 0.1mL of the crude enzyme from the culture and the mixture was incubated in water bath at 60°C for 10 min. The reaction was stopped with 3.5mL of 30mM NaOH. Finally, 0.5mL of 0.02% (w/v) phenolphthalein in 5mM Na2CO3 was added and mixed well. After leaving the mixture to stand for 15min at room temperature, the reduction in colour intensity was measured at 550nm. A blank lacking the enzyme is tested simultaneously with each batch of samples. One unit of enzyme activity was defined as the amount of enzyme that forms 1µmol of β-CD from soluble starch in 1 min.
Construction of standard graph for Cyclodextrin

Stock solution

100mg of β-CD was dissolved in minimal quantity of distilled water in a 10mL volumetric flask and made the volume up to the mark with distilled water, which resulted 10mg/mL. 1mL of the above solution was taken in a volumetric flask of 10mL and distilled water was added to make up to 10mL, which resulted 1mg/mL. It was used as a standard stock solution.

From the above solution, 1mL was taken in a volumetric flask of 10mL and made the volume up to the mark which resulted in 100µg/mL.

Procedure

Into a series of 10mL volumetric flask 1.0mL, 2.0mL, 3.0mL, 4.0mL and 5.0mL of standard stock solution of β-CD was taken and distilled water was added to make up to 10mL mark in each volumetric flask, mixed well and optical density was measured at 550nm after developing the colour as described above, against a reagent blank prepared in same manner. The data is shown in the Table 3.1.

A standard curve was constructed taking concentration of β-CD (µg/mL) on X-axis and corresponding optical density on Y-axis shown in the Fig 3.1.

OPTIMIZATION OF PROCESS PARAMETERS BY CONVENTIONAL METHOD

Inoculum/ Production medium

Medium I (Gawande and Patkar 2001)

Soluble starch 1.0%
Yeast extract 0.5%
Peptone 0.5%
Na₂HPO₄ 0.1%
Materials and Methods

MgSO₄ 0.02%
Distilled water up to 100mL
pH 7.0

Preparation of Inoculum

For the preparation of inoculum, the growth contents of two days old slant culture was suspended into 5mL of sterile distilled water and transferred into 45mL of inoculum medium contained in 250mL Ehrlenmeyer flask. The flask was incubated on a rotary shaker at 30ºC for 24h. 1% of this suspension was transferred to production medium.

Preparation of Production medium

1% (0.5mL) of the above inoculum medium was transferred aseptically to 49.5mL of production medium. The flasks were kept on the rotary shaker at 30ºC. The samples were withdrawn for every 12h up to 72h and centrifuged at 10,000rpm for 10min in order to remove the cells and other insoluble materials. The clear supernatant was used as a crude enzyme for estimation.

Optimization of process parameters

Effect of initial pH on CGTase production

In order to understand the influence of initial pH of the medium on CGTase production the medium was adjusted to pH 5.5 to 9.5 with 1N HCl and 1N NaOH. The flasks were inoculated with the 1mL of 24h old culture and kept for incubation at 30ºC for 48h. The samples were withdrawn and analysed for the CGTase activity.

Effect of incubation temperature on CGTase production

The influence of incubation temperature on CGTase production by the isolated bacteria was investigated by incubating the medium in various temperature ranges from 20 to 40ºC.
Effect of inoculum size and age on CGTase production

To study the influence of inoculum size on enzyme production the medium flasks were incubated with various concentrations of inoculum from 0.5 to 3.5mL of 24h culture. To investigate the effect of age of inoculum 18 to 48h old culture was inoculated in the media. After incubation, the samples were withdrawn and estimated for CGTase activity.

Effect of various carbon and nitrogen source on CGTase production:

In order to study the best suitable carbon source for effective CGTase production by the isolated *Bacillus* sp TPR71H various carbon compounds viz soluble starch, xylose, lactose, maltose, sucrose, glucose, mannitol, fructose and galactose were studied at 1%. While in case of nitrogen source, various organic and inorganic nitrogen compounds such as peptone, casein, yeast extract, malt extract, ammonium chloride, ammonium sulphate, ammonium phosphate and sodium nitrate were investigated at 0.5%. Further the best suitable compounds were studied at various concentrations.

Effect of incubation time on CGTase production

Finally, experiments were run under the optimized conditions obtained from the above experiments. The flasks were incubated upto 72h and the samples were withdrawn periodically for every 12h and analysed for the CGTase production.

All experiments were conducted in triplicate and the mean values were considered.

STRAIN IMPROVEMENT STUDIES

Microorganism

Initially the cultural and process parameters were optimized and the best producing strain of CGTase was selected. This strain was used for strain improvement studies to further increase CGTase production. The strain was used for UV irradiation. The best strain derived from UV treatment was used for HNO$_2$ treatment and the best
strain obtained from this treatment was further used for EMS exposure. All these strains were maintained on nutrient agar slants. Generally an 18h old culture was used for the preparation of inoculum.

**Preparation of reagents**

**Phosphate buffer (pH 6.5)**

Sodium dihydrogen phosphate (15.6g) and disodium hydrogen phosphate (17.8gm) were weighed and added to distilled water and made up to the final volume of 1L.

**Acetate buffer (0.1 M, pH 4.2)**

Sodium acetate (8.4g) was weighed and added to glacial acetic acid (3.35mL). The solution was made up to a final volume of 1L of distilled water.

**Sodium nitrite (0.01M)**

Sodium nitrite (13.6g) was weighed and dissolved in 1L water to give concentration of 0.01M.

**Sodium hydroxide (0.1 N)**

Sodium hydroxide (4g) was weighed and dissolved in 1L of distilled water to give a 0.1N solution.

**Preparation of inoculum**

An 18h old culture of the isolated strain on nutrient agar slant was made into suspension with 5mL of sterile distilled water and transferred into 45mL of sterile nutrient broth in a 250mL conical flask. The flask was incubated on a rotary shaker for 24h. From this 5mL of inoculum was transferred to a 45mL of fresh sterile nutrient broth and again incubated on a rotary shaker for 24h. The broth was transferred into sterile centrifugation tube aseptically, centrifuged at 3000rpm for 15min. The supernatant was decanted and the cell mass was resuspended in sterile phosphate buffer and centrifuged.
The supernatant was decanted and 40mL of sterile phosphate buffer was added and made into suspension. This suspension was used for UV irradiation.

**Methods**

**UV Irradiation**

The prepared suspension was transferred aseptically in 4mL quantities into previously labeled 10cm diameter sterile petriplates having flat bottoms. The exposure to UV rays was carried out in ‘Dispensing Cabinet’ fitted with TUP 40 W germicidal lamp, which emits 90% of its radiation at 2540-2550°A. The exposure was carried out for time periods like 30, 60, 90, 120, 150, 180 and 210sec. UV lamp was stabilized previously for 30min. During the UV exposure the lid of the petriplates were removed and occasional shaking was done. During the UV exposure all other sources of light were cut off and the exposure was carried out in dim light and the exposed suspensions were kept in dark area for dark repair. This has been done to avoid photo reactivation. Each irradiated suspension was serially diluted and plated with the nutrient agar and incubated at 37°C for 24h. After 24h of incubation, 18 isolates were selected on the basis of morphology, size and shape of the colony. The selected colonies were picked up, evaluated for their CGTase production capacities and mutant which showed maximum CGTase production was selected for subsequent mutation and subjected to nitrous acid treatment.

**Nitrous acid treatment**

The cell suspension was prepared as described earlier. Acetate buffer of pH 4.2 was used. To 18mL of the cell suspension in buffer, 2mL of sterile stock solution of 0.01M sodium nitrite was added. Samples were withdrawn at 20, 40, 60 and 80min. Each sample was neutralized with 1mL of 0.1N sodium hydroxide, serially diluted and plated on the nutrient agar medium. The plates were incubated at 37°C for 24h. The colonies
were selected on the basis of morphology, size and shape. Selected colonies were subcultured on to the nutrient agar slants.

**EMS treatment**

The cell suspension was prepared as described earlier. 1mL of cell suspension was added to 5mL of EMS solution (1mg/mL) and the resulting solution was incubated at 37°C. Two mL of this solution was taken at 60, 120, 180 and 240 min and centrifuged. Pellet obtained was serially diluted and plated with nutrient agar medium. The plates were incubated at 37°C for 24h. The colonies were selected on the basis of morphology, size and shape. Selected colonies were subcultured on to the modified nutrient agar slants (Akhund and Khvostova, 1966).

**OPTIMIZATION OF PROCESS PARAMETERS BY TAGUCHI METHOD**

**Microorganism and Culture Conditions**

In the present study a mutated *Bacillus* sp. TPR71H (Gen Bank No: FN993946) was used. This culture was stored in a nutrient agar slants and subcultured periodically once a week.

**Optimization by using Taguchi method**

Taguchi methodology for optimization can be divided into four phases, viz. planning, conducting, analysis and validation. Each phase has a separate objective and contributes towards the overall optimization process. Taguchi methodology for optimization has been represented in the form of a flowchart as shown in Fig 3.2.

**Phase I—Planning**

Phase 1 is the first step, this determines the various factors to be optimized in the culture conditions that have critical effect on the CGTase yield. Based on the preliminary studies, five fermentation factors viz pH, initial temperature, agitation speed, time of incubation and inoculum concentration which are having significant influence on the
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enzyme yield, were selected for the present study. A Taguchi experimental design was employed to optimize these factors. In the present study a 16 experimental orthogonal array (OA) was used. All five factors were chosen at four levels. Table 3.2 depict the selected process parameters and their levels and Table 3.3 depicts the L16 Taguchi experimental plan. In the design each column consisted of a number of conditions depending on the levels assigned to each factor.

Phase II – Conducting the Experiments

Mutated bacterial strain was incubated in 100 mL of medium containing starch 2% (w/v), yeast extract 0.5% (w/v), K$_2$HPO$_4$ 0.5% (w/v) and MgSO$_4$ 0.04% (w/v). The process parameters such as pH, inoculum size, agitation speed, incubation temperature and time were varied according to the Taguchi design. After a specific time as per experimentation the flasks were removed from the shakers and the culture was centrifuged at 10,000rpm for 10min to remove the biomass and other insoluble substrates from the culture. After centrifugation the supernatant liquid was collected and estimated for CGTase activity.
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Objective of the study

Identification of control factors and their levels

Phase I (Planning)

Design the matrix experiments

Conducting the experiments

Phase II (Conducting)

Data Analysis

Phase III (Analysis)

Optimum levels determination for factors

Validation experiments

Phase IV (Validation)

Fig 3.2. Experimental protocol of DOE for CGTase production

by mutated *Bacillus* sp. TPR71H NA6
Phase III—Analysis of experimental data (AED)

The experimental data obtained was processed using Qualitek-4 software with bigger-is-better quality characteristics for the determination of the optimum culture conditions for the enzyme yield; to identify individual factor influence on the CGTase production; and to estimate the performance (fermentation) at the optimum conditions. In Taguchi’s method, quality is measured by the deviation of a characteristic from its target value and a loss function \( L(y) \) is represented by

\[ L(y) = k \cdot (y - m)^2 \quad (2) \]

Where \( k \) = the proportionality constant,

\( m \) = the target value and

\( y \) = the experimental value obtained for each trial.

In case of bigger-is-better quality characteristics, the loss function can be written as

\[ L(y) = k \cdot \frac{1}{y^2} \quad (3) \]

And the expected loss function can be represented by

\[ E [L(y)] = k \cdot E \left( \frac{1}{y^2} \right) \quad (4) \]

Where \( E \left( \frac{1}{y^2} \right) \) can be estimated from a sample of \( n \) as

\[ \frac{\sum_{i=1}^{n} \frac{1}{y_i^2}}{n} \quad (5) \]

Phase IV—Validation of experiment

In order to validate the methodology, fermentation experiments were further performed for CGTase production, using the obtained optimized culture conditions.
OPTIMIZATION OF NUTRIONAL FACTORS BY PLACKETT-BURMANN DESIGN

Microorganism and Culture Conditions

In the present study a mutated *Bacillus* sp. TPR71H (Gen Bank No: FN993946) was used. This culture was stored in a nutrient agar slants and subcultured periodically once a week. The production of CGTase experiments were conducted according to the PBD. The liquid samples are withdrawn and centrifuged at 10,000rpm for 10min to remove the biomass and other insoluble substrates from the culture. After centrifugation the supernatant liquid was collected and estimated for CGTase activity.

Estimation of CGTase activity

Enzyme activity was measured by decrease of phenolphthalein colour intensity. Enzyme assay was carried out according to the Kaneko *et al.*, 1987 method. The reaction mixture containing 1mL of 40mg of soluble starch in 0.1M potassium phosphate buffer (pH 6.0) and 0.1mL of the crude enzyme from the culture was incubated in water bath at 60°C for 10min. The reaction was stopped with 3.5mL of 30mM NaOH. Finally, 0.5mL of 0.02% (w/v) phenolphthalein in 5mM Na₂CO₃ was added and mixed well. After leaving the mixture to stand for 15min at room temperature, the reduction in colour intensity was measured at 550nm. A blank lacking the enzyme is tested simultaneously with each batch of samples. One unit of enzyme activity was defined as the amount of enzyme that forms 1µgm of β-CD from soluble starch in 1min.

Plackett–Burman Design (PBD)

Various carbon and nitrogen compounds required for the effective CGTase production by mutated *Bacillus* sp. TPR71H NA6 were selected based on Plackett-Burman design. Table 3.4 shows the selected compounds and their levels. All the compounds were chosen at three levels. In these levels the concentration of each
compound was fixed based on the literature survey and our own experience gained. Table 3.5 depicts the experimental PB experimental plan. Analysis of the experimental results was performed based on the effect of each variable. The effect of the each selected variable on CGTase production was determined based on the first-order model

\[ Y = \beta_0 + \sum \beta_i x_i \] (1)

Where \( Y \) is the response (enzyme activity), \( \beta_0 \) is the model intercept, \( \beta_i \) is the linear coefficient, and \( x_i \) is the level of the independent variable. The significance of each variable was determined based on ANOVA. All experiments were conducted in triplicates and averages of the results were taken as the response.

**OPTIMIZATION OF PROCESS VARIABLES BY RESPONSE SURFACE METHODOLOGY**

**Microorganism and culture conditions**

In the present study a mutated *Bacillus* sp. TPR71H (Gen Bank No: FN993946) was used. This culture was stored in nutrient agar slants and subcultured periodically once every week. The production of CGTase experiments were conducted according to the PBD. The liquid samples are withdrawn and centrifuged at 10,000rpm for 10min to remove the biomass and other insoluble substrates from the culture. After centrifugation the supernatant liquid was collected and estimated for CGTase activity.

**Optimization by Response surface methodology (RSM)**

Response surface methodology using Central composite design was applied for optimization of CGTase production from mutated *Bacillus* sp. TPR71HNA6. Four important parameters namely starch (X1), yeast extract (X2), K2HPO4 (X3), and MgSO4 (X4) concentrations were selected as the independent variables and the enzyme activity (CGTase activity U/mL) was the dependent response variable. Each of these independent
variables was studied at five different levels as per CCD in four variables with a total of 28 experimental runs. CGTase activity (U/mL) corresponding to the combined effects of four variables was studied in their specified ranges as shown in Table 3.6. The process variables such as temperature, pH and agitation speed were kept constant throughout the experiment. All the flasks were analysed for CGTase activity at the end of the experiment. The plan of CCD in the coded levels of the four independent variables is shown in Table 3.7.

For statistical calculations the independent variables were coded as

\[
x_{i} = \frac{(X_{i} - X_{0})}{\delta X_{i}} = x_{i}
\]

Where \(X_{i}\) is the experimental value of variable; \(X_{0}\) is the midpoint of \(X_{i}\), \(\delta X_{i}\) is the step change in \(X_{i}\) and \(x_{i}\) is the coded value for \(X_{i}\), \(i = 1–4\).

This response surface methodology allows the modelling of a second order equation that describes the process. CGTase production data was analysed and response surface model given by Eq. (2) was fitted with multiple regressions through the least squares method.

\[
Y_{i} = \beta_{0} + \sum_{i=1}^{k} \beta_{i}x_{i} + \sum_{i=1}^{k} \beta_{ii}x_{i}^{2} + \sum_{i=1}^{l} \sum_{j=1}^{l} \beta_{ij}x_{i}x_{j}
\]

Where \(Y_{i}\) is the predicted response, in the present study CGTase production (\(Y_{i}\)) taken as a response, \(x_{i}\) \(x_{j}\) are input variables which influence the response variable \(Y\); \(\beta_{0}\) is the offset term; \(\beta_{i}\) is the \(i^{th}\) linear coefficient; \(\beta_{ii}\) the \(i^{th}\) quadratic coefficient and \(\beta_{ij}\) is the interaction coefficients

**Data analysis and Interpretation of the results**

The results of the experimental design were analysed and interpreted using the STATISTICA version 7.0 (Statsoft, USA) statistical software. Prediction of optimum
fermentation parameters and shape of the curves generated by the model was also done by the same software.

IMMOBILIZATION TECHNIQUES

Microorganism

A mutated strain of *Bacillus* sp. TPR71HNA6 was used for the present study.

Preparation of cell suspension

A 50mL of cell suspension was prepared by adjusting the total number of cells to 4.2x10^7 cfu/mL as described earlier and was used for immobilization in various matrices.

1. ADSORPTION TECHNIQUE

Preparation of supporting matrices for adsorption technique

Polyurethane foam

Polyurethane foam was weighed and washed thoroughly with distilled water. It was added to the conventionally optimised production medium at a final concentration of 2% (w/v) and sterilized by autoclaving.

Glass Wool

Glass wool was treated with concentrated nitric acid for 3h and washed with distilled water. Then it was added to the selected production medium (Medium I) at a final concentration of 2% and sterilized by autoclaving.

Cotton Cloth

Cotton cloth, procured from the local market was used as supporting matrix for the immobilization. The cloth was cut in to small pieces (0.5cm^2). One gram (2% w/v) of these cloth pieces were taken into petridishes and washed with distilled water to remove the soluble compounds that may be present in the cloth and then the cloth pieces were squeezed to remove the absorbed water. Cotton pieces were transferred in to 45mL of
selected production medium (Medium I) contained in 250mL Ehrlenmeyer flasks, sterilized by autoclaving and cooled.

**Absorbent Cotton**

The absorbent cotton was selected to immobilize the mutated strain *Bacillus* sp. TPR71HNA6. A thin layer of absorbent cotton was cut into small pieces (0.5cm²). One gram (2% w/v) of these cotton pieces were taken into petridishes and washed with distilled water to remove the soluble compounds that may be present in the cloth and then the cotton pieces were squeezed to remove the absorbed water. The pieces were transferred into 45mL of selected production medium (Medium I) contained in 250mL Ehrlenmeyer flasks, sterilized by autoclaving and cooled.

**Fermentation with immobilized cells and free cells**

Five mL of inoculum (4.2x10⁷ cfu/mL) of mutated *Bacillus* sp. TPR71HNA6 was added separately to 45mL of fermentation medium containing 2% w/v of supporting matrix. The flasks were incubated at 30°C on a rotary shaker (220rpm) for 5 days. A set of six Ehrlenmeyer flasks were employed in triplicate and one flask was harvested for every 24h and subjected for CGTase activity.

**Repeated batch fermentation**

In repeated batch fermentations, the fermentation medium was decanted from each flask at every 96h and the supporting matrix with immobilized cells was washed twice with 50mL of sterile saline solution. Then a fresh medium (50mL) was added to each flask and the fermentation was continued for next cycle. This process was repeated for all succeeding cycles.
Chapter III Materials and Methods

2. ENTRAPMENT TECHNIQUE

Preparation of supporting matrices for entrapment technique

Sodium alginate solution

Sodium alginate solution (3%) was prepared by dissolving sodium alginate in 100mL of hot distilled water. The contents were stirred vigorously for 10min to obtain thick uniform slurry without any undissolved lumps and sterilized by autoclaving.

Calcium chloride solution

A liter volume of calcium chloride in concentrations of 0.2M was prepared in distilled water. The solution was stored at room temperature.

k-Carrageenan

k-Carrageenan was completely dissolved in distilled water (3g/100mL) at the desired concentration at 50°C, then the solution was sterilized at 110°-121°C for 1h. The solution was cooled to < 38-40°C with stirring.

Entrapment of cells in calcium alginate

Entrapment of cells in alginate is one of the simplest, cheapest, less toxic and the most frequently used method of immobilization (Palmieri et al., 1994; Park and Khang 1995; Bikerstaff, 1997). The cell suspension was added to prepared alginate slurry and stirred for 10min to get uniform mixture. The slurry was taken into sterile syringe, added drop wise into 0.2M calcium chloride solution from 5cm height to form calcium alginate beads. The beads were kept for curing at 4°C for 1h. The cured beads were washed with sterile water for 3-4 times. The beads were preserved in normal saline solution at 4°C. All these operations were carried out aseptically under laminar airflow unit (Farid et al., 1994).
**Entrapment of cells in k-Carrageenan**

The cell suspension was added to molten k-Carrageenan solution maintained at 40°C, mixed well and poured into sterile flat bottomed 10mm dia petriplates. After solidification it was cut into equal size cubes (4x4x4mm) and added to sterile 2% potassium chloride solution and kept in the refrigerator for 1h for curing. The cubes were washed 3-4 times with sterile distilled water (Veelkan and Pape, 1982).

**Entrapment of cells in Agar-Agar**

A definite quantity of agar-agar was dissolved in 18mL of 0.9% sodium chloride solution to get the final concentration of 2% w/v and sterilized by autoclaving. Then 2mL of cell suspension was added to the molten agar-agar maintained at 40°C, shaken well for few seconds, poured into sterile flat bottomed 10mm dia petriplates and allowed to solidify. The solidified agar block was cut into equal size cubes (4x4x4mm) added to the sterile 0.1M disodium phosphate buffer (pH 6.5) and kept in refrigerator 1h for curing. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water for 3-4 times.

**Entrapment of cells in Gelatin**

2mL of the cell suspension was added to 15mL of 15% sterile gelatin solution maintained at 45°C and poured into sterile petriplate. The gel was over layered with 10 mL of glutaraldehyde for hardening at 30°C. The resulted blocks was cut into small cubes (4x4x4mm) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde.

**Production of CGTase by batch process with immobilized cells**

The immobilized beads/ cubes were transferred into 50mL selected production medium (Medium I) contained in 250mL Ehrlenmeyer flasks. The flasks were incubated
at 30°C for 72h. Samples were withdrawn at regular intervals of 12h and assayed as described earlier.

**Production of CGTase by repeated batches**

The fermentation production medium with immobilized beads/cubes was conducted for 96h for the succeeding batches, where maximum productivity was attained. The medium was replaced with fresh production medium and the process was repeated for several batches until the beads/cubes started disintegrating. The CGTase titres for each cycle were evaluated as described earlier.

**PURIFICATION TECHNIQUE**

All enzyme purification steps were carried out at 0 to 4°C.

**Ammonium Sulphate Precipitation**

The crude broth obtained after fermentation was centrifuged at 6000rpm for 10min to remove the cell biomass. Solid ammonium sulphate was added slowly to the culture supernatant to get 60% saturation, stirred for 60min and left for overnight at 4°C. The precipitate was harvested by centrifugation at 10,000 X g for 10 min, dissolved in 0.1M acetate buffer (pH 5.5) and dialyzed against same buffer overnight (4°C). The dialyzed sample was then assayed for CGTase activity and protein content.
Separation of CGTase by affinity chromatography

Preparation of affinity matrix

The matrix was prepared according to the Sian et al., 2005. Five grams of Sepharose 4B was thoroughly washed with distilled water. The gel was then transferred to 20mL coupling solution (0.1M NaOH) containing of 350mg β-cyclodextrin and reacted for 16h at 45°C. After incubation, the gel was washed again with distilled water for 1h. Subsequently, the washed gel was transferred to methanolamine solution (pH 8.0) and was incubated for 24h at 50°C. This step is essential to block any unreacted epoxy groups. The gel was then washed with 0.1M acetate buffer pH 4.0 followed by 0.1M Tris–HCl buffer pH 8.0 containing 0.5M NaCl. This cycle was repeated for three times. Finally, the gel was packed into a 15mm X 100mm column and equilibrated with two bed volumes of 0.1M sodium acetate buffer pH 5.5.

Affinity chromatography

The dialyzed enzyme mixture was loaded onto a 15mm X 100mm β-CD-Sepharose 4B affinity column that was previously equilibrated with the same buffer at a flow rate of 25mL/h. The column was washed with 0.01M acetate buffer pH 5.5 for 4h. Elution of the bound enzyme was carried out with the same buffer supplemented with 1% β-CD. All fractions were collected and each one was assayed for CGTase activity and protein content. All CGTase purification procedures were performed at 4°C.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), according to LaemmLi, 1970. A 15% cross linked polyacrylamide gel on a Tarson gel electrophoresis unit (Tarson, India) was used for the present study. Electrophoresis was carried out at constant voltage...
(300V and 60mA) for 90min at room temperature. Coomassie Brilliant Blue (0.1%) staining was used to detect the protein bands on the gel.

**Characterization of CGTase**

**Effect of substrate concentration on CGTase activity**

To observe the effect of substrate on CGTase the enzyme concentration was kept constantly (0.1mL) and the various concentrations of starch (1 to 6g/L) were used. The CGTase activity was observed at different time intervals for each concentration of starch.

**Determination of optimum pH and temperature**

The optimum pH of the pure enzyme was determined by replacing 0.1M phosphate buffer pH 6.0 in the CGTase assay with the following buffers: citrate buffer, 0.1M (pH 4 to 6), phosphate buffer, 0.1M (pH 5 to 8), borate buffer, 0.1M (pH 8 to 10) and bicarbonate buffer (pH 9 to 11). The reaction was carried out using the CGTase assay procedure mentioned above. The optimum temperature of the pure enzyme was determined by performing the CGTase assay in different temperatures, ranging from 20° to 90°C at pH 6.0.

**Determination of optimum pH and temperature by means of RSM**

In order to determine the optimum pH and temperature of CGTase a central composite design was employed. These factors were coded at five levels starting from -1.414, –1, 0, 1, and 1.414 by using Eq. 1.

\[
x_i = \frac{X_i - X_0}{\Delta X_i}
\]

Where

\(x_i\) is the dimensionless coded value of the variable \(X_i\), \(X_0\) the value of the \(X_i\) at the centre point, and \(\Delta X_i\) the step change.
For statistical calculations, the variables \( X_i \) were coded as \( x_i \) according to Equation 1. The range and levels of the variables in coded units for RSM studies were reported in Table 3.8. The behaviour of the system was explained by the following quadratic model 2.

\[
Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad \text{(2)}
\]

Where

- \( Y \) is the predicted response,
- \( \beta_0 \) the intercept term,
- \( \beta_i \) the linear effect,
- \( \beta_{ii} \) the squared effect, and
- \( \beta_{ij} \) the interaction effect.

In the present investigation, 10 experimental central composite experimental design was used to determine the optimum response of pH and temperature of CGTase produced by the *Bacillus* sp. MATLAB 7.0 (Mathworks USA) software was used for regression and graphical analysis of the data obtained.

**Determination of pH and thermal stability**

The pH stability of the enzyme was measured by incubating 0.1mL pure enzyme with 0.2mL phosphate buffer, 0.1M (pH 5 to 8) and borate buffer, 0.1M (pH 8 to 9) respectively at 60°C, without substrate for 30min. The remaining activity of the enzyme was assayed by the standard assay method. The temperature stability of the enzyme was measured by incubating 0.1mL pure enzyme with 0.2mL buffer (0.1M phosphate buffer, pH 6.0) without substrate at different temperatures (60°C–80°C) for 30min. Then the enzyme was cooled to room temperature and standard CGTase assay was performed to determine their residual activity.
The effects of metal ions and reagents on CGTase activity

In this study, 0.1mL of purified enzyme was mixed with 0.2mL of 0.1 M phosphate buffer pH 6.0, containing different metals and reagents at 1 mM & 2mM (final concentration) and incubated for 10min at room temperature. The standard CGTase assay was performed to determine the activity.

Estimation of Kinetic parameters

The $K_m$ and $V_{max}$ values for the pure enzyme were determined by incubating 0.1mL of purified CGTase in 1mL 0.1 M phosphate buffer pH 6.0 at various concentrations of soluble starch solution, ranging from 1 to 6g/L at 60°C for 10min. The values of $K_m$ and $V_{max}$ were then determined by using the Graph pad Prism V 4.0 software. The activation energy was calculated using Arrhenius plot which was plotted $\ln V$ vs $1/T$ (K).
Table 3.1. Construction of standard graph for β- CD

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<th>β- CD concentration (μg/mL)</th>
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y = 0.002x
R² = 0.999

β-cyclodextrin Concentration (μg/mL)

Fig 3.1: Standard graph for β-cyclodextrin (β-CD)
Table 3.2. Selected Bioprocess parameters and their assigned Levels

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<th>S. No</th>
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<td>Temperature (°C)</td>
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<td>Agitation speed (rpm)</td>
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<td>Incubation time (hrs)</td>
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Table 3.3. L-16 Taguchi experimental plan with coded values

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<th>X4</th>
<th>X5</th>
<th>CGTase activity (U/mL)</th>
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Table 3.4. Factors in coded and real values used for screening of significant components by PBD

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<td>10</td>
<td>Ammonium sulphate</td>
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Table 3.6. Experimental range and coded levels of process variables for CGTase production

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<td>MgSO$_4$ (X4)</td>
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Table 3.7. Lay out of Full factorial central composite design for CGTase production by mutated *Bacillus* sp. TPR71HNA6

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<th>Starch (X1)</th>
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Table 3.8. Layout of central composite design for determination of pH and temperature optimum

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