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# ISOLATION OF CYCLODEXTRIN GLUCANOTRANSFERASE PRODUCING ALKALOPHILIC BACTERIA AND OPTIMIZATION OF CULTURE CONDITIONS FOR ITS PRODUCTION

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## ABSTRACT

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) producing alkalophilic bacteria were isolated using starch agar plates (pH >10.0) containing phenolphthalein and methyl orange as an indicator. Nine different soil samples were screened for CGTase producing bacteria and five isolates were obtained. An alkaliphile AND3 showed the highest CGTase production (1.99 U/ml) among the isolates obtained, so it was selected for the further study. Physical culture conditions like inoculum size, 2 % (v/v), temperature, 37 °C and $Na_2CO_3$ (1 %, w/v) were found optimum for CGTase production. Different carbon sources like soluble starch, refined wheat flour, rice flour and sago flour were tested for CGTase production and rice flour (6.77 U/ml) was proved as the best raw carbon source. Among the organic (peptone, yeast extract) and inorganic nitrogen sources (ammonium sulphate, ammonium nitrate, ammonium chloride, sodium nitrate) tested, ammonium sulphate showed the highest enzyme (7.4 U/ml) production. Optimization of culture conditions resulted to 3.7 fold increase in CGTase production. CGTase was partially purified by ammonium sulphate fractionation and has shown 113 U/mg specific activity, 4.85 fold purification with 60 % yield. Partially purified enzyme showed the best activity at 50 °C temperature and 6.0 pH.

**KEY WORDS:** **Cyclodextrin glucanotransferase, starch, flour, alkaliphile, optimization**

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# INTRODUCTION

Starch is an important storage compound synthesized by many plants as their carbon and energy source. Many bacteria can utilize different starch substrates as their carbon and energy source. Cyclodextrins (CDs) are produced as a result of intramolecular transglycosylation (cyclization) reaction during degradation of starch by cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) enzyme. The CGTase is a multifunctional enzyme, catalyzing four different reactions: cyclization, disproportionation (cleavage of a linear maltooligosaccharide and transfer to a linear acceptor oligosaccharide), coupling (opening of the rings of CDs and transfer to a linear acceptor oligosaccharides), and weak hydrolysis reaction (production of a linear maltodextrin).[1] Cyclodextrins are cyclic oligosaccharides commonly composed of six, seven, or eight D-glucose units (α-, β-, and γ-cyclodextrins, resp.) joined by α 1,4-glycosidic bonds.[1] A torus shaped microring of cyclodextrin molecule is having hydrophilic hydroxyl (-OH) groups on the outside of the ring molecule with the hydrophobic -CH groups and glycosidic oxygen located inside the cavity of the molecule. Because of this unique property, CDs can form molecular inclusion complexes with range of compounds and hence have found various applications.[2,3] CDs can alter the solubility of a guest compound, stabilize against the effect of light, heat, and oxidation, mask unwanted physiological effects, and reduce volatility as a result of molecular complexation phenomenon. CDs are used in many industrial productions, analytical methods, pharmaceuticals, food and flavors, cosmetics, packing, textiles, separation processes, and so forth.[3,4] β-Cyclodextrin and its derivatives are the most widely used form of cyclodextrins as it is commercially available and inclusion complexes can be prepared easily with β-CD.[5] CGTase is an extracellular enzyme, predominantly produced by strains of *Bacillus*, namely, *B. circulans* 251[2], *B. macerans*[6], *B. firmus*[7], *Bacillus stearothermophilus* HR1[8], *Bacillus sp.* TS1[9] etc. Majority reports of CGTase production using bacteria noticed β-cyclodextrin as their major product in a mixture of other CDs in different ratios. Production of microbial metabolites can be increased by manipulating nutritional requirements, physical parameters, and genetic make-up of the producing strain.[10] Selection and incorporation of appropriate carbon, nitrogen, and other nutrient sources play an important role in designing the cost-effective production medium. CGTase has been commonly produced by submerged fermentation in media containing various types of starch or other carbohydrates and complex nitrogen sources. The aim of the present work was to isolate novel alkalophilic bacteria for CGTase production and optimization of its culture conditions to maximize the enzyme production.

# MATERIALS AND METHODS

### *Chemicals*

Soluble Starch, β-cyclodextrin, yeast extract, peptone, phenolphthalein and sodium carbonate were purchased from Merck specialities private limited, India. Ammonium sulphate and Methyl-Orange were bought from Sloca Research laboratories Pvt. Ltd. Agar powder was purchased from HiMedia laboratories Pvt. Ltd. Finely grounded rice flour and sago flour were collected from local flour mills and were sieved through fine cheese cloth to get fine uniform raw starch powder. Refined wheat flour (maida) was bought from local market. All other chemicals used were of analytical grade.

### *Soil sample collection and preparation of suspension*

CGTase producing bacteria were isolated from different soil samples of Anand district of Gujarat, India. The ten different garden soil, fertile farm soil and saline soil samples were collected. To prepare the soil suspension, approximately 1.0 gm of each soil sample was added in a 10 ml sterile distilled water tube and was vigorously shaken. Then, the suspended soil particles were allowed to settle down and supernatant was used as suspension.

### *Isolation of CGTase producing bacteria*

Ten different soil samples were screened for isolation of CGTase producing alkalophilic bacteria using spread plate method. Prepared soil suspension was diluted appropriately and 0.1 ml of each soil suspension was spreaded over the starch agar plates containing (g/L) starch, 10; peptone, 5; yeast extract, 5; $K_2HPO_4$, 1; phenolphthalein, 0.3; methyl orange, 0.1; agar, 20; $Na_2CO_3$, 1 (autoclaved separately); pH >10.0. Inoculated plates were incubated at 37 °C and checked regularly for colonies showing a yellowish halo surrounding its growth up to 72 h.[8] Colonies showing the clear zone were carefully transferred on to slant culture containing the above mentioned medium except dyes and maintained at 4 °C by periodic transfer.

### *Morphological and cultural characteristics*

The morphology of selected bacterial isolates were studied by Gram's staining and motility test was carried out by hanging drop preparation.

### *Preparation of Bacterial inoculum*

Pure culture from slant was inoculated in 50 ml basal medium containing (g/L) starch, 10; peptone, 5; yeast extract, 5; $K_2HPO_4$, 1; $Na_2CO_3$, 10 (autoclaved separately) in 250ml Erlenmeyer flask. The inoculated flasks were incubated at 37 °C, 150 rpm for 24 h. Cells were then harvested in sterile centrifuge tubes (15 mL) at 5000 rpm for 10 min. Pellet of cells was resuspended in sterile medium to get an optical density (OD) of 1.0 at 660 nm and used as an inoculum.

### *CGTase production from natural isolates*

After primary screening, four isolates were selected for further study on the basis of zone of clearance produced on starch agar plates with indicator dyes. Four bacterial isolates namely AND1, AND3, AND5 and AND7 were tested for CGTase production using shake flask culture. 100 mL production medium was prepared in 250 mL flasks and sterilized at 121 °C for 15 minutes. The pH of media was adjusted to alkaline range (>10.0) by adding separately sterilized $Na_2CO_3$. Inocula were prepared for these isolates and sterilized flasks were inoculated. They were incubated at 37 °C, 150 rpm on a rotary shaker for 72 h. Media flasks were harvested after 72 h, cells and suspended particles were removed by

centrifugation and the supernatant was used to determine CGTase activity.

***CGTase assay***

CGTase activity was determined by phenolphthalein assay method described by Goel and Nene[7] with minor modification. 100 µL approximately diluted enzyme was incubated with 1.0 mL of 50 mg soluble starch in sodium phosphate buffer (pH 6.0, 0.05 M) at 60°C for 30 min. The reaction was stopped by quickly cooling the tubes on ice. Four milliliters of working phenolphthalein solution was added, the tubes were vortexed, and the absorbance of the mixture was immediately measured at 550 nm. The working phenolphthalein solution was made by adding 1 mL of phenolphthalein stock (4 mM in ethanol) to 100 mL of 125 mM $Na_2CO_3$ prepared in 4% ethanol. The standard β-cyclodextrin estimation was also carried using the same method. One unit was defined as the amount of enzyme that produced one µmole of β-cyclodextrin per min. The amount of β-CD produced was estimated from a standard curve of 0 – 100 µg/ml concentration. One CGTase unit was defined as the amount of enzyme that produced one µmole of β-CD per min under assay conditions. Protein concentration was determined by Folin-Lowry's method with bovine serum albumin (0-100 µg) as standard.

***Effect of different inoculum size***

To check effect of inoculum size (% v/v) on CGTase production, 2%, 3% and 5% inoculum was added in 50 ml of basal medium in 250 ml flask and incubated at 37°C, 150 rpm on a rotary shaker. CGTase was estimated at an interval of 24 h for 48 h.

***Optimization of $Na_2CO_3$ concentration***

The selected isolate AND3 is highly alkalophilic in nature and even could not grow well at pH less than 10.0. In this set of experiment, concentration of $Na_2CO_3$ (% w/v) was tested as a parameter. Four flasks containing 50 ml basal medium were prepared, sterilized and then, separately autoclaved $Na_2CO_3$ was added at different concentrations of 0.5%, 1.0%, 2.0% and 3.0%. They were inoculated and incubated at 37 °C, 150 rpm on a rotary shaker for 48 h and CGTase production was analysed.

***Effect of carbon sources on CGTase Production***

In a basal medium, 1.0 gm% soluble starch was supplied as a major carbon source. To check the effect of various carbon sources on CGTase production, soluble starch was replaced with different carbon sources viz. rice flour, sago flour and refined wheat flour. Four flasks containing 50 ml basal medium with different starch sources were inoculated and incubated at 37 °C, 150 rpm on a rotary shaker for 48 h. After incubation, cells were separated by centrifugation and CGTase production was measured from each flask.

***Effect of nitrogen sources on CGTase Production***

Different organic (yeast extract, peptone) and inorganic nitrogen (ammonium sulphate, ammonium nitrate) sources were tested for CGTase production using AND3. Organic nitrogen sources were added at 0.5 gm% concentration and inorganic nitrogen sources were added at 0.3 gm%. Four flasks containing 50 ml basal medium with different nitrogen sources were inoculated and incubated at 37 °C, 150 rpm on a rotary shaker for 48 h. After incubation, cells were separated by centrifugation and CGTase production was measured from each flask. As isolate AND3 could utilize inorganic nitrogen sources, in subsequent set of experiment more inorganic sources viz. ammonium nitrate, ammonium sulphate, ammonium chloride and sodium nitrate were tested at 0.3 gm% for CGTase production. In this set, rice flour was added in the basal medium to replace soluble starch as it was found as the best carbon source. Four flasks containing 50 ml basal medium with rice flour as carbon source and different inorganic nitrogen sources were inoculated and incubated at 37 °C, 150 rpm on a rotary shaker for 48 h. After incubation, cells were separated by centrifugation and CGTase production was measured from each flask.

***Ammonium sulphate precipitation***

The optimized medium containing 1.0 gm% rice flour, 0.3 gm% $(NH_4)_2SO_4$, 0.1 gm% $K_2HPO_4$, 1.0 gm% $Na_2CO_3$ (autoclaved separately) was inoculated with 2% (v/v) inoculum of AND3. Inoculated culture flask was incubated at 37°C, 150 rpm for 48 h. After incubation, cells were removed by centrifugation. Supernatant was used for ammonium sulphate precipitation. 90 ml of the crude enzyme broth was brought to 0-20% saturation with slow addition of solid ammonium sulphate in an ice bath with gentle stirring. This flask was kept at 4 °C for 2 h. The precipitated proteins were separated by centrifugation at 8°C, 8000g for 15 min. The resulted protein pellet was dissolved in phosphate buffer (100 mM, pH 6.0). The supernatant was further precipitated with ammonium sulphate to achieve 20-70% saturation, in ice bath with continuous gentle stirring. The flask was kept at 4 °C overnight. Next day, proteins of second fraction was separated by centrifugation at 8 °C, 8000g for 15 min. The second protein pellet obtained was dissolved in phosphate buffer (100 mM, pH 6.0) and stored at 4°C until further use. Precipitated proteins were carefully transferred to a dialysis bag using a micropipette and dialyzed against phosphate buffer (100 mM, pH6.0) at 4°C. The buffer was stirred gently using a magnetic stirrer to enhance solute exchange. Dialysis was carried out overnight and the buffer was changed several times to increase the efficiency of the dialysis. Amount of CGTase enzyme and protein was estimated from crude as well as protein precipitate fractions. The specific activity and fold purification was calculated for partially purified CGTase from AND3.

***Effect of temperature and pH on CGTase activity***

The effect of temperature on partially purified CGTase was determined at different temperature 0f 40°C, 50°C, 60°C and 70°C. Appropriately diluted 100 µl of enzyme was added in 1.0 ml of 50 mg soluble starch in sodium phosphate buffer (pH 6.0, 100 mM) and incubated for 10 min at different temperatures. After incubation, CGTase activity was measured. The effect of pH on partially purified CGTase activity was determined using different pH phosphate buffers (100 mM) i.e. 5.5, 6.0, 7.0 and 8.0. Appropriately diluted 100 µl of enzyme was added in 1.0 ml of 50 mg soluble starch in different pH buffers and incubated for 10 min at 50 °C. After incubation, CGTase activity was measured.

## RESULTS AND DISCUSSION

***Isolation of CGTase producing bacteria***

The isolation of CGTase producing bacteria from soil was carried out using starch agar plate with phenolphthalein and methyl orange as described by Park *et al*.[8] Incorporation of phenolphthalein and methyl orange in medium gives red colour at pH >10. Starch was degraded by the CGTase producing bacteria from the surrounding area of colonies. In this area where cyclodextrins are produced, medium lost its colour and a yellowish zone was observed due to inclusion complex formation between cyclodextrins and dyes. Total nine isolates were obtained which has produced yellow zone surrounding the colonies after 48 h (Fig. 1). Out of these, four cultures were found potent CGTase producer and they are further screened by submerged production using shake flask culture.

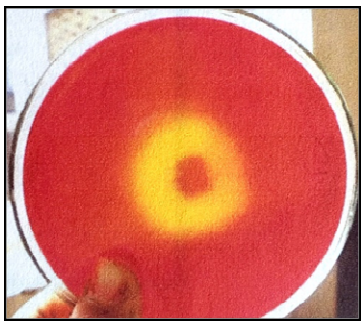

**Figure 1**
***Yellow zone of clearance produced by isolate AND3***

***Morphological study of isolates***

Gram's staining was done to determine the basic morphological characteristics of these four bacterial cultures and motility test was carried out by hanging drop preparation. All four bacteria were Gram positive, medium to big rod and motile.

***CGTase production and selection of bacterial culture***

Four bacterial cultures viz. AND1, AND3, AND5 and AND7 were selected for further CGTase production on the basis of zone of clearance produced. They were inoculated in 50 ml basal medium in 250 ml flask with 2% (v/v) inoculum and incubated at 37°C, 150 rpm on a rotary shaker for 72 h. Two milliliters of sample was withdrawn at every 24 h from each flask, cells were separated by centrifugation and appropriately diluted supernatant was analysed for CGTase production. The CGTase production of these cultures is shown in table 1. The bacterial isolate AND3 produced the highest enzyme (1.99 U/ml) after 48 h.

**Table 1**
***CGTase production in shake flask culture***

| Bacterial culture | CGTase (U/ml) | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| AND1 | 0.68 | 1.69 | 1.40 |
| AND3 | 1.19 | 1.99 | 1.05 |
| AND5 | 0.47 | 1.18 | 0.99 |
| AND7 | 0.55 | 1.23 | 0.81 |

***Effect of different inoculum size***

On the basis of above results, two cultures AND1 and AND3 found better with 1.69 U/ml and 1.99 U/ml CGTase production, respectively. They were further screened for inoculum size and tested with different inoculum size of 2%, 3% and 5% in 50 ml of basal medium in 250 ml flask. Inoculated flasks were incubated at 37°C, 150 rpm on a rotary shaker for 48 h. The inoculum size of 2% (v/v) was found as the best for CGTase production for AND1 (1.62 U/ml) and AND3 (1.95 U/ml). An alkaliphile AND3 was selected for the further studies.

**Table 2**
***Effect of inoculum size on CGTase production***

| Bacterial culture | CGTase (U/ml) | | |
|---|---|---|---|
| | 2 % (v/v) | 3 % (v/v) | 4 % (v/v) |
| AND1 | 1.62 | 1.22 | 0.58 |
| AND3 | 1.95 | 1.84 | 0.99 |

***Optimization of $Na_2CO_3$ concentration***

As selected isolate AND3 is highly alkalophilic, influence of sodium carbonate concentration was checked for CGTase production. The highest enzyme production of 2.51 U/ml was occurred with 1.0 gm% $Na_2CO_3$ in basal medium (Table 3).

**Table 3**
***Effect of $Na_2CO_3$ concentration on CGTase Production***

| $Na_2CO_3$ (% w/v) | CGTase (U/ml) |
|---|---|
| 0.5 | 1.80 |
| 1.0 | 2.51 |
| 2.0 | 0.69 |
| 3.0 | 0.68 |

***Effect of carbon sources on CGTase production***

Along with soluble starch, different raw carbon sources like rice flour, sago flour and refined wheat flour (maida) were tested for enzyme production. The isolate AND3 could grow and produced reasonably good quantity of CGTase with all the tested raw starches (Table 4). Among the carbon source tested, rice flour gave the highest production of 6.77 U/ml after 48 h. The alkalophilic *Bacillus stearothermophilus*HR1 and *Bacillus* sp. TS1-1 showed better CGTase production with 1.5 gm% and 1.48 gm% of sago starch, respectively.[9,10] Ibrahim *et al.*[12] reported the highest CGTase from *Bacillus* G1 with tapioca starch [11].Maximum CGTase production by *B. circulans* DF 9R was obtained with 1.5 gm% cassava starch.

**Table 4**
***Effect of carbon sources on CGTase production***

| Carbon sources | CGTase (U/ml) |
|---|---|
| soluble starch | 1.78 |
| Refined wheat flour (maida) | 5.11 |
| Rice flour | 6.77 |
| Sago flour | 0.78 |

***Effect of nitrogen sources on CGTase Production***

Among the tested nitrogen sources, ammonium sulphate influenced significantly on the CGTase production than yeast extract, peptone and ammonium nitrate with AND3 (Table 4). The highest CGTase production (7.40 U/ml) was observed with ammonium sulphate at 0.3 gm% concentration after 48 h. As like ours, CGTase production from *B.circulans* DF 9R showed ammonium sulphate as the best nitrogen source.[12] Rahman *et al*.[9] also reported maximum production of CGTase from *B.stearothermophilus* HR1 with 2 gm% peptone. The highest CGTase production from alkalophilic *Bacillus* sp. TS1-1 was reported at 1.89 gm% yeast extract.[10] Ibrahim *et al*.[11] investigated the influence of organic and inorganic nitrogen sources on CGTase production from *Bacillus* G1 and they found peptone as the best nitrogen source.

**Table 5**
***Effect of nitrogen sources on CGTase production***

| Nitrogen sources | CGTase (U/ml) |
|---|---|
| Yeast extract | 3.51 |
| Peptone | 3.89 |
| Ammonium sulphate | 7.40 |
| Ammonium nitrate | 6.00 |

**Table 6**
***Effect of inorganic nitrogen sources on CGTase production***

| Inorganic nitrogen sources | CGTase (U/ml) |
|---|---|
| Ammonium nitrate | 5.98 |
| Ammonium sulphate | 7.36 |
| Ammonium chloride | 3.92 |
| Sodium nitrate | 0.63 |

***Ammonium sulphate precipitation***

The crude CGTase enzyme produced by AND3 bacterial isolate was concentrated and partially purified by ammonium sulphate precipitation. Two fractions of 0-20% and 20-70% ammonium sulphate were carried out. CGTase activity and protein estimation from crude, 0-20% and 20-70% fractions were done. In 0-20% fraction, specific activity of 53.72 U/mg and 2.28 fold purification was observed whereas in 20-70% fraction enzyme specific activity was 113.92 U/mg with 4.85 fold purification. Total yield of CGTase enzyme in both fraction of ammonium sulphate precipitation was 60.35%.

**Table 7**
***Partial purification of CGTase by ammonium sulphate precipitation***

| Procedure | Activity (U/ml) | Protein (mg/ml) | Specific activity (U/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Crude enzyme | 4.6 | 0.20 | 23.46 | 1 | 100 |
| Ammonium sulphate (0-20%) | 10.96 | 0.21 | 53.72 | 2.28 | 60.35 |
| Ammonium sulphate (20-70%) | 25.52 | 0.23 | 113.92 | 4.85 | |

**Effect of temperature and pH on CGTase activity**

The parameters like temperature and pH have very high influence on enzyme activity. The effect of temperature on enzyme activity was measured over a temperature range of 40 °C to 70 °C. The best enzyme activity was observed at 50 °C temperature and the other tested temperatures showed decrease in enzyme activity. The CGTase from an alkaliphilic *B. agaradhaerens*strain LS-3C exhibited an optimum temperature of 55 °C.[13] Most of the CGTases reported have optimum temperature between 50 and 65 °C for their activity.[14] The effect of pH on CGTase activity was characterized at pH 6.0, 7.0 and 8.0 (100 mM, Phosphate buffer). The results indicated that the optimum pH of 6.0 found as the best for enzyme activity. The optimal pH value obtained is in accordance with other reported pH values in the literature. The CGTases from *Microbacterium terrae* KNR 9 and *Bacillus* sp. G1 have shown the highest enzyme activity at 6.0 pH.[14,15]

## CONCLUSION

Five CGTase producing isolates were sereened from nine different soil samples. An alkaliphile AND3 selected for study showed the highest CGTase production of 1.99 U/ml. Upon optimization of physical culture conditions, CGTase production was increased to 6.77 U/ml using raw starches like rice flour. AND3 could grow in presence of inorganic nitrogen ammonium sulphate and produced 7.4 U/ml CGTase resulted in 3.7 fold increase. Selected AND3 isolate can be further explored for CGTase production using raw starches and inorganic nitrogen sources.

## CONFLICT OF INTEREST

Conflict of interest declared none.

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