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Catalysis Research

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Review

Microbial Cyclodextrin Glycosyltransferases: Sources, Production, and Application in Cyclodextrin Synthesis

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Academic Editor: Pedro Fernandes

Special Issue: Recent Trends in Biocatalysis

Catalysis Research
2022, volume 2, issue 3
doi:10.21926/cr.2203029

Received: June 11, 2022
Accepted: September 07, 2022
Published: September 22, 2022

Abstract

Cyclodextrin glycosyltransferase (CGTase) is a multifunctional enzyme that hydrolyzes the *α*-glycosidic bond between two sugar molecules and synthesizes cyclodextrins (CDs) and other transglycosylation products. It is a ubiquitously present extracellular enzyme that offers the CGTase-producing organism the sole right onto starch substrates over other microbes. The present review provides a brief account of diversity among CGTase-producing microbes, CGTase production in different heterologous hosts (wherein extracellular secretion is highly desired), and different physicochemical properties of CGTases. Overall, 52 crystal structures that highlight the five domain tertiary structure of CGTases have been discovered so far. On the basis of these structures, the catalytic mechanism of CGTase reactions has been discussed, and three catalytic residues, namely Glu257, Asp229, and Asp328, have been identified at the active site in all CGTases. Moreover, the active site is constituted by at least nine sugar-binding sites, denoted as -7 to +2. Furthermore, a sequence alignment of selected CGTases highlighted the conserved regions and the sequential differences among *α*-CGTases, *β*-CGTases, and *γ*-CGTases. Various biotechnological applications of CGTases and CGTase

immobilization on a variety of support matrices are briefly discussed. This review also encompasses a detailed account of CDs, their enzymatic production, extraction, and applications in different industrial sectors.

Keywords
Cyclodextrin glycosyltransferase; structure; immobilization; enzymatic synthesis; application

1. Introduction

The enzyme cyclodextrin glycosyltransferase (abbreviated as CGTase; EC 2.4.1.19) is a part of the glycosyl hydrolase (GH) superfamily within the GH13_2 subfamily. The enzymes *α*-amylase and maltogenic amylases also belong to this class of enzymes, and all three enzymes share substantial sequence similarities (http://www.cazy.org). All three enzymes possess three conserved domains A, B, and C, whereas CGTases have two more domains D and E [1-5]. The multifunctional CGTases are mainly considered transferase enzymes due to their ability to catalyze various transglycosylation reactions (such as coupling, disproportionation, and cyclization); however, it also exhibits a minor hydrolysis activity [6, 7]. Of these reactions, cyclization is the major activity of CGTases and involves the cleavage of starch or similar oligosaccharides at the *α*-1,4-glycosidic bond leading to the formation of a glycosyl-intermediate. The reaction is followed by the formation of a new intramolecular *α*-1,4-glycosidic bond at the non-reducing end of the substrate, resulting in the formation of cyclodextrins [6].

The commercial applications of CGTases include the production of cyclodextrins (CDs) and various transglycosylated products [5, 7]. Cyclodextrins are oligosaccharides that are arranged in a cyclic structure linked together through *α*-1,4-glycosidic bonds and are mainly categorized on the basis of the number of glucose residues involved in their structure [8-10]. Naturally occurring cyclodextrins include *α*-CDs, *β*-CDs, and *γ*-CDs consisting of six, seven, and eight glucose units, respectively [8-10]. These CDs are arranged uniquely as truncated cones, with an internal hydrophobic cavity and a hydrophilic outer surface that allows the formation of an inclusion complex with a variety of hydrophobic molecules. Because of this ability, CDs are widely employed in various industrial fields, including food, pharmaceuticals, cosmetics, plastics, and agrochemical industries [2, 11, 12]. The synthesis of CDs is a much-explored field, with industrial production being performed by the enzymatic conversion of starch by using microbial CGTases. Extracellular CGTase secretion has been reported in various microbes, including bacteria, archaea, and fungi [5, 13, 14]. CGTase-catalyzed CD production results in a mixture of *α*-CDs, *β*-CDs, and *γ*-CDs; therefore, CGTases are classified as *α*-CGTases, *β*-CGTases, or *γ*-CGTases on the basis of the ratio of the formed product [15]. CGTases from different strains of the genus *Bacillus* have been well-characterized, with most of them being *β*-CGTases, followed by *α*-CGTases, and *γ*-CGTases [5, 13]. All wild-type CGTases produce a mixture of CDs, including *α*-CDs, *β*-CDs, *γ*-CDs, and large-ring CDs [16, 17].

As evident from the current vast CD applications, the demand for CD and its application-based products will elevate to nearly $390 million by 2027 (https://www.gminsights.com/industry-analysis/global-cyclodextrin-

market?utm_source=globenews%20wire.com&utm_medium=referral&utm_campaign=Paid_globenewswire). Currently, only two commercial CGTases are available in the market. The first one is Toruzyme (CGTase from *Thermoanaerobacter* sp. ATCC 53627), which produces a mixture of CDs and is marketed by Novozyme, Denmark. The second is Amano (CGTase from *Paenibacillus macerans*), which also produces a mixture of CDs and is marketed by Amano Enzyme Europe Ltd. (Milton, UK). Therefore, processes for the large-scale production of CGTases should be developed. Although several literature reports and reviews are available on the enzyme CGTase and its applications in various fields, the present review summarizes the available information on various aspects of CGTase and its applications, such as CD production, over the last 15 years.

2. Sources and Diversity of Microbial CGTase

CGTase is a ubiquitously present extracellular enzyme in nature and is produced by various microorganisms. CGTases are largely produced by eubacteria (Table 1); however, few archaea, such as *Thermococcus* sp. strain B1001 [18], *Pyrococcus kodakaraensis* [19], *Pyrococcus furiosus* DSM 3638 [20], and *Haloferax mediterranei* [21], have also been reported to produce CGTase. Fungal CGTases have been reported only from *Trichoderma viride* [22]. Most CGTases are *β*-CD producers (*β*-CGTases), followed by *α*-CGTases and *γ*-CGTases.

Table 1 List of selected CGTase-producing bacteria.

Microorganism	Major CD produced	References
Klebsiella pneumoniae AS-22	*α*	Gawande and Patkar [23]
Thermoactinomyces vulgaris Tac-5354	*α*	Abelyan et al. [24]
Bacillus clarkii 7364	*γ*	Takada et al. [25]
Anaerobranca gottschalkii	*α*	Thiemann et al. [26]
Bacillus circulans A11	*β*	Rimphanitchayakit et al. [27]
Bacillus sp. G-825–6	*γ*	Hirano et al. [28]
Paenibacillus campinasensis H69–31	*α/β*	Alves-Prado et al. [29]
Bacillus megaterium	*β*	Pishtiyski et al. [30]
Paenibacillus graminis NC2213	*α/β*	Vollú et al. [31]
Thermoanaerobacter sp. P4	*β*	Avci et al. [32]
Bacillus sp. TS1–1	*β*	Rahman et al. [33]
Bacillus firmus 7B	*β*	Moriwaki et al. [34]
Paenibacillus sp. T16	*β/γ*	Charoensakdi et al. [35]
Amphibacillus sp. NPST-10	-	Ibrahim et al. [36]
Bacillus thuringiensis GU2	*γ*	Goo et al. [37]
Microbacterium terrae KNR 9	-	Rajput et al. [38]

3. Physiological Significance of CGTase Production

Some microorganisms secrete CGTases into their extracellular environment. CGTase then utilizes the starch substrate present outside the cells to produce CDs through its cyclization activity. These CDs are then taken up by these microorganisms through specific translocation machinery. Inside the cells, these CDs are utilized as an energy source by modifying them into

various oligosaccharides, which are then taken up in the carbon utilization pathways, such as glycolysis. This ensures that the microorganisms secreting CGTase have the sole right over the starch substrate, which cannot be utilized by competing microorganisms; the majority of such organisms have cyclodextrin hydrolase activity as well [39].

4. Production and Properties of CGTase

CGTase is a starch-inducible extracellular enzyme produced by several microorganisms of high industrial significance (Table 1). However, CGTase production from wild-type organisms results in lower yields that [39, 40] can be enhanced using various strategies, such as culture medium optimization through a stepwise approach or statistical media optimization approach [36, 38, 41-49]. Moreover, enzyme overproduction can be achieved through heterologous enzyme expression [7, 50]. Various reports have also demonstrated CGTase expression on the cell surface of various hosts. Wang et al. [51] reported the cloning of CGTase from *B. circulans* 251 in pYD1 plasmid and its subsequent expression on the surface of *Saccharomyces cerevisiae*, thereby allowing the yeast cells to utilize starch as the sole carbon source and produce CDs as the major products together with glucose and maltose.

Heterologous production strategies can improve enzymatic expression through protein engineering, and codon optimization of genes based on the host organism [52]. Other recombinant hosts are also used for CGTase production, such as *Bacillus subtilis* and *Pichia pastoris*, because they offer extracellular secretion together with higher yields [7, 50]. Among various heterologous expression hosts, *E. coli* is the most popular host for CGTase expression because of its easy and rapid cultivation, low cost of enzyme production, high protein yields, and easy system for foreign gene expression [50, 53]. Heterologous expression of *CGTase* from *Paenibacillus* sp. T16 in *E. coli* JM109 cells produced similar levels of CGTase in a pH 7 culture medium after 24 h of culture time compared with wild-type CGTase produced under longer culture time (72 h) conditions and in a pH 10 culture medium [35]. Moreover, gene codon bias also limits CGTase expression in heterologous hosts, which can be compensated using codon optimization or the supplementation of rare codons by using a pRARE plasmid. Lee et al. [20] reported the successful expression of CGTase from *Pyrococcus furiosus* DSM3638 in *E. coli* after alleviating codon bias by using a pRARE plasmid. The purified CGTase exhibited optimum activity at pH 5 and 95 °C, thermostability even at 100 °C, and converted starch to *β*-CD majorly [20]. Song et al. [54] reported the cloning of codon-optimized CGTase encoding gene (*my20*) procured from the metagenome sequencing of marine microorganisms (obtained from Mariana Trench) in a pET24a vector and its subsequent expression in *E. coli* BL21 (DE3). Recombinant CGTase was maximally expressed at 20 °C after 18 h postinduction by using 0.4 mM IPTG, with the enzyme exhibiting optimal activity at pH 7 and 80 °C [54].

However, a major bottleneck in the overexpression of enzymes in an *E. coli* system is the formation of inclusion bodies [40, 55]. The expression of soluble enzymes can be further enhanced by employing various strategies, including changing the fermentation conditions, such as the cultivation media change together with medium composition change [56]. Moreover, coexpression of chaperons like GroEL-GroES, DnaJ-GrpE [57, 58] and conventional approaches like changes in growth pH, temperature, salt, and agitation can improve enzyme production in *E. coli* [59, 60]. Recombinant production of CGTase from *Paenibacillus pabuli* US132 in *E. coli* increased (1

U/mL to 22 U/mL) upon reducing the postinduction temperature from 37 °C to 19 °C and employing a 2TY medium for expression instead of LB medium [61]. Similarly, Liu et al. [62] reported the maximum expression of CGTase from *Bacillus* sp. T1 at 25 °C postinduction with 0.3 mM IPTG and OD_{600} = 0.8 after 10 h incubation in *E. coli* BL21 (DE3) host. Moreover, Duan et al. [63] optimized recombinant production of *γ*-CGTase from *B. clarkii* 7364 by using the Placket-Burman and response surface methodologies in *E. coli* BL21 (DE3) host. They reported a 2.8-fold enhancement in *γ*-CGTase activity (53992 U/mL; hydrolysis activity) after medium optimization [63]. Kim et al. [64] reported a 6-fold enhancement in the soluble expression of *B. macerans* CGTase in *E. coli* upon the coexpression of both DnaK-DnaJ-GrpE and GroEL-GroES. In addition, the coexpression of human peptidyl-prolyl cis-trans isomerase (PPIase) maximally enhanced (14-fold) the soluble expression of CGTase [64]. The use of appropriate promoters upstream of the cloned gene improved enzyme yields in some studies [7, 39].

Extracellular enzyme production may improve recombinant enzyme yields by omitting the cell lysis and sonication steps, thereby reducing the overall cost. To achieve extracellular protein secretion, various native secretion signals and signal peptides, such as PelB sequence, sec signal, and L-asparaginase, are utilized [65-67]. Ong et al. [68] reported successful expression and secretion of CGTase from *Bacillus* sp. G1 in *E. coli* by using the native signal peptide that converted starch into 90% *β*-CD and 10% *γ*-CD. In addition, other methods of codon optimization, site-directed mutagenesis, appropriate promoter selection, and expression in different hosts, such as *Bacillus subtilis* and *Pichia pastoris,* are usually employed to increase recombinant protein production [7, 50]. Jeang et al. [69] compared the expression and characterization of CGTase from native *B. macerans* host, with the same protein expressed in *B. subtilis* and *E. coli* hosts. All three exhibited similar enzymatic properties; however, the protein expressed in *E. coli* had lower thermostability and nearly 14-fold higher *β*-CD coupling activity than the native CGTase and CGTase expressed in *B. subtilis* [69]. CGTase expression in heterologous hosts has been reviewed by several studies [7, 50], with variable production yields being reported. On the basis of the available literature from the last decade, a list of selected reports on heterologous CGTase expression is presented in Table 2.

Table 2 List of reports on extracellular CGTase expression in heterologous hosts.

CGTase source	Strategy	Host	Remarks	Reference
P. macerans strain JFB05–01	*CGTase* cloned in pET20b+ vector downstream of a pelB signal peptide; culture conditions were optimized	*E. coli* BL21 (DE3)	Extracellular secretion of CGTase with an activity of 22.5 U/mL was achieved after 90 h of induction by using 0.01 M IPTG in TB medium at 25 °C	Li et al. [70]
	CGTase cloned in pET20b+ vector downstream of a pelB signal peptide; evaluated the effect of glycine on extracellular secretion		Maximum secretion of *α*-CGTase was achieved after the addition of 1% glycine in the TB medium at the middle of the exponential phase; enzyme activity in the culture medium was highest (2.6 U/mL to 28.5 U/mL) after 44 h	Li et al. [71]
	CGTase cloned in pET20b+ vector downstream of a pelB signal peptide; evaluated the effect of various medium additives on extracellular secretion		The presence of 0.03% SDS, 400 mM Na^+, 0.3% glycine, and 10 mM Ca^{2+} in TB medium enhanced extracellular secretion, with an enzyme activity of 12.9 U/mL (15-fold higher than the absence of additives)	Ding et al. [72]
	Codon optimized gene cloned in pET20b+ vector having a pelB signal peptide		The overall yield of codon-optimized protein (4230 mg/L) was higher than those of proteins expressed without codon optimization (1710 mg/L)	Liu et al. [73]
B. clarkii 7364	Effects of temperature, chemical chaperones, and inducers on cell growth and *γ*-CGTase production	*E. coli* BL21 (DE3)	Total yield of soluble *γ*-CGTase with the addition of *β*-CD in the culture medium (50.3 U/mL) was 1.7-fold higher than the absence of *β*-CD (29.3 U/mL)	Wang et al. [74]
P. macerans	Codon optimized gene cloned under inducible P_{xyl} promoter	*B. megaterium* MS941	Maximum *α*-CGTase activity of 48.9 U/mL was achieved after 24 h of production at 32 °C	Zhou et al. [75]

	and the signal peptide SP_{LipA}; cultivation parameters were optimized			
P. pabuli US132	Extracellular CGTase production from *B. subtilis* by cloning *CGTase* under lipase signal peptide; culture parameters were optimized	*E. coli*	Maximum CGTase production of 9.27 U/mL was achieved	Ayadi-Zouari et al. [76]
B. stearothermophilus	Screening signal peptides	*B. subtilis* WS11	Replacement of several amino acids from the N-terminus with appropriate signal peptide led to a 2.3-fold increase in extracellular *α*/*β*-CGTase activity in a 3L fermenter	Su et al. [77]
Bacillus sp. G1	*CGTase* cloning in a single and dual plasmid system (promoter selection) aided with a hemolysin transport system; selection of a better host; optimization of cultivation parameters	*E. coli* BL21 (DE3), Rosetta 2 (DE3), Rosetta blue (DE3), and Rosetta-Gami 2 (DE3)	Extracellular CGTase activity of 69 U/mL was achieved in BL21 (DE3) host by using a dual plasmid system after parameter optimization	Low et al. [78]
B. lehensis G1	Recombinant *E.coli* cells (*CGTase* cloned with M5 signal sequence) immobilized on hollow fiber membranes	*E. coli* BL21 (DE3)	The immobilized cells exhibited a 2.8–4.6-fold increase in extracellular secretion over free cells	Man et al. [79]
	Proteome-based identification of signal peptides for efficient extracellular secretion	*E.coli* BL21 (DE3)	GlcNAc-binding protein A (GAP) signal peptide enhanced CGTase activity in the extracellular fraction (735%) than native signal CGTase peptide	Ling et al. [80]
Bacillus sp. G825–6	Codon optimized gene cloned in pET20b+ with three sec-type	*E.coli* BL21 (DE3)	DacD signal peptide increased the extracellular CGTase activity with a 7.3-fold enhancement over	Sonnendecker et al. [81]

	signal peptides (PelB, DacD, and native CGTase signal)		native CGTase signal after 24 h of induction	
Bacillus sp. NR5 UPM	Optimization of parameters, such as inducers (glycine, triton X-100, and xylose), time, and temperature	*E. coli* JM109	Extracellular *β*-CGTase activity increased 1.3-fold to 38.29 U/mL (than the control without glycine) postinduction with 1.2 mM glycine at 37 °C within 12 h	Nik-Pa et al. [82]
	Codon optimization and glycine supplementation		Extracellular *β*-CGTase activity increased 2.2-fold to 65.52 U/mL (than the control without glycine) postinduction with 1.2 mM glycine at 37 °C within 12 h	Nik-Pa et al. [83]
B. stearothermophilus NO_2	Three point mutants (I631T, I641T, and K647E) were generated by directed evolution	*E. coli* BL21 (DE3)	Point mutant K647E resulted in a 2.2-fold enhancement in CGTase production over wild-type enzyme; the yield further increased to 1904 U/mL in a 3L fermenter	Tao et al. [84]
B. firmus strain 37	Heterologous expression	*B. subtilis* WB800	Enzyme activity of recombinant CGTase was 7.4-fold higher than the wild strain	Gimenez et al. [85]
B. circulans STB01	Heterogeneous expression and site-directed mutagenesis, Y89G/D577R, Y89D/D577R, and Y89N/D577R	*B. subtilis* WB600	Double mutant Y89D/D577R exhibited the highest *β*-cyclization activity and *β*-CD production	Huang et al. [86]
Bacillus sp. SD5	Mature *CGTase* sequence was cloned in pPICZ*α*A vector with gene only (N2), with c-myc epitope and polyhistidine tag from plasmid (S3), and with polyhistidine tag from primer (M5)	*Pichia pastoris* X-33/*Komagataella phaffii*	Presence of additional tags did not affect enzyme production; all three clones exhibited maximum enzyme activity after 24 h production. For M5 clone, K_m and V_{max} values were 13.59 mg/mL and 1153 μmol/(mg min)	Kabacaoglu and Karakas Budak [87]
B. pseudalcaliphilus	Codon optimization; selection of		*β*-CGTase activity was maximal (3885.1 U/mL) at	Zhang et al.

8SB	appropriate expression vector and culture condition optimization	28 °C, 6% inoculum ratio, and 1.5% methanol addition after 24 h of incubation	[88]
	Culture media parameters were optimized	*β*-CGTase activity was increased to 122 U/mL in the modified medium (from no enzyme activity)	Zhang et al. [89]

All known CGTases are monomeric and their molecular weights vary from 64 to 90 kDa according to the source of origin [5, 50]. However, CGTase from an alkaliphilic bacterium *Microbacterium terrae* KNR 9 has the smallest molecular weight of 27.7 kDa [38], and CGTase from *Bacillus agaradhaerens LS-3C* has the highest molecular weight of approximately 110 kDa [90]. Most CGTases from different *Bacillus* sp. have temperature optima within the range of 40–65 °C [38, 50, 91]. However, various thermostable CGTases optimally function at higher temperatures (60 °C and above). For example, CGTase from *Thermoanaerobacter thermosulfurigenes* EM1 has a temperature optimum of 80–85 °C [92], CGTase from *Thermoanaerobacter* sp. has a temperature optimum of 80 °C [54], and CGTase from *Bacillus stearothermophilus* NO_2 has an optimum enzyme activity at 70 °C [84]. Moreover, recombinant CGTase from *Pyrococcus furiosus* DSM3638 expressed in *E. coli* exhibited a temperature optimum of 95 °C [20]. CGTases from several *Bacillus* sp. are stable within a temperature range of 40–70 °C [38, 91]. Various CGTases exhibit a pH optimum around neutral pH [30, 38, 91, 93, 94], whereas some others have an alkaline pH optimum, for example, CGTases from *Bacillus* sp. strain G-825–6 [28], *E. clarkii* [25], and *Brevibacterium* sp. strain 9605 [95]. In addition, CGTases are stable within a neutral to alkaline pH range [5].

5. Structure of CGTase

To date, 52 crystallized CGTase structures of bacterial origin are available in the Research Collaboratory for Structural Bioinformatics Protein Database (RCSB PDB) (search term: CGTase). Of these, 31 CGTases are from *Niallia circulans*, 11 are from *Bacillus* sp., 4 are from *Thermoanaerobacterium thermosulfurigenes* and *Paenibacillus macerans*, and 1 is from *Geobacillus stearothermophilus* and *Evansella clarkii*. A total of 30 CGTases that are listed below (Table 3) have been crystallized in the last 22 years (2000–till date).

Table 3 List of selected CGTase PDB structures (from 2000 till date).

CGTase source	PDB Id	Type of CGTase	Resolution (Å)	Remarks	Ligands*	Release year	Reference
Paenibacillus macerans	6L2H	*α*	2.10	CGTase mutant-Y167H	CA	2019	-
	6AIJ		2.10	CGTase mutant-N603D	CA	2018	-
	3WMS		2.30	CGTase mutant-Y195I	CA	2014	Xie et al. [96]
	4JCL		1.70	Wild CGTase	CA, CL, EDO, GOL, PEG, PGE	2014	-
Evansella clarkii	4JCM	*γ*	1.65	Wild CGTase	CA, CL, EDO, GOL, NA, SO4	2014	-
Thermoanaerobacterium thermosulfurigenes	3BMW	*β*	1.60	CGTase mutant S77P complexed with a maltoheptaose inhibitor	ACI, CA, CL, GOL, SO4	2008	Kelly et al. [97]
	3BMV		1.60	CGTase mutant S77P	CA, GOL, SO4	2008	
Bacillus sp. 1011	1V3M	*β*	2.00	CGTase mutant F283Y complexed with pseudotetraose derived from acarbose	ACI, CA, GAL, GLC	2004	Kanai et al. [98]
	1V3L		2.10	CGTase mutant F283L complexed with pseudotetraose derived from acarbose	ACI, CA, GLC	2004	
	1V3K		2.00	CGTase mutant F283Y	CA	2004	
	1V3J		2.00	CGTase mutant F283L	CA	2004	
	1UKT		2.20	CGTase mutant Y100L complexed with acarbose	ACI, CA	2004	Haga et al. [99]
	1UKS		1.90	CGTase double mutant F183L/F259L complexed with pseudomaltotetraose	ACI, CA, GLC	2004	

	1UKQ		2.00	Wild CGTase complexed with pseudomaltotetraose	ACI, CA, GLC	2004	
	1PJ9		2.00	Loop mutant between 183 and 195	ACY, CA, GLC, MPD	2004	Leemhuis et al. [100]
	1PEZ		2.32	CGTase mutant A230V	ACY, CA, EPE, MPD	2003	Leemhuis et al. [101]
Niallia circulans	1OT1	*β*	2.00	CGTase mutant D135A	ACY, CA, EPE, MPD	2003	Leemhuis and Dijkhuizen [102]
	1OT2		2.10	CGTase mutant D135A	ACY, CA, EPE, GLC, MPD	2003	
	1KCL		1.94	CGTase mutant G179L	CA, GLC, MPD	2002	Leemhuis et al. [1]
	1KCK		2.43	CGTase mutant N193G	ADH, CA, GLC	2002	
Bacillus sp. 1011	1I75	*β*	2.00	Wild CGTase complexed with 1-deoxynojirimycin	CA, NOJ	2001	Kanai et al. [103]
Niallia circulans	1EO7	*β*	2.48	Wild CGTase complexed with maltohexaose	CA	2000	Uitdehaag et al. [104]
	1EO5		2.00	Wild CGTase complexed with maltoheptaose	CA, MPD	2000	
Bacillus sp. 1011	1DED	*β*	2.00	CGTase mutant H233N complexed with acarbose	CA	2000	Ishii et al. [105]
	1D7F		1.90	CGTase mutant H233N	CA	2000	
Niallia circulans	1DTU		2.40	CGTase mutant Y89D/S146P complexed with hexasaccharide inhibitor	ADH, CA	2000	Van der Veen et al. [6]

*CA, CL, EDO, GOL, PEG, PGE, NA, SO4, ACI, GLC, ACY, MPD, EPE, ADH, and NOJ represent calcium ion, chloride ion, 1,2-ethanediol, glycerol, polyethylene glycol, phenyl glycidyl ether, sodium ion, sulfate ion, 6-amino-4-hydroxymethyl-cyclohex-4-ene-1,2,3-triol, alpha-D-glucopyranose, acetic acid, (4S)-2-methyl-2, 4-pentanediol, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, 1-amino-2,3-dihydroxy-5-hydroxymethyl cyclohex-5-ene, and 1-deoxynojirimycin, respectively.

5.1 Domain Specification

CGTases are monomeric proteins with five domains and catalyze four types of reactions, namely coupling, hydrolysis, cyclization, and disproportionation [5, 14]. The first report on the 3D structure of CGTase in 1991 [106] provided key insights into its tertiary structure and revealed that all CGTases consist of five domains, A, B, C, D, and E. Of these, A, B, and C domains are common to all α-amylase family enzymes, whereas D and E domains are unique to CGTases only. Moreover, A and B are catalytic domains, and C and E bind to substrates, such as starch granules; however, the role of domain D remains unknown, with a few studies suggesting hydrolytic activity [5, 7, 14, 107]. The active site is located at the bottom of an eight (β/α) barrel-like structure in the A domain [5, 14]. The barrel-like arrangement is formed by the highly symmetrical fold of eight parallel β-strands that are encircled by eight α-helices [5, 7]. Domain B is a protuberant loop between β-strand 3 and α-helix 3 of domain A and contains 44–133 amino acid residues that play crucial roles in substrate binding [5, 6]. Goh et al. [108] demonstrated a calcium-binding site at the A/B domain interface that could significantly influence the stability of CGTase by introducing a new salt bridge at the protein surface in domain B. Substrate binding occurs in a long groove formed by the A and B domains on the surface of an enzyme that can fit in at least 10 glucose residues (seven at the donor subsites and three at the acceptor subsites, respectively; labeled as -7 to +3), as revealed by kinetic studies and crystal structures of substrate/inhibitor/product-CGTase complexes [5, 14, 102]. Three domains (C, D, and E) constitute the C-terminal region in CGTases and exhibit a β-sheet structure [39]. The D and E domains are the characteristics of all CGTases [5, 7, 14]; the sequence of domain D is similar to that of the IPT/TIG domain, and its importance in CGTases remains unexplored. The E domain or the raw-starch binding domain, belongs to the family 20 of carbohydrate-binding modules (CBM20, http://www.cazy.org), with the presence of two maltose-binding sites [7, 109].

5.2 Catalytic Residues

The analysis of various CGTase crystal structures revealed that CGTase has three active site residues, Glu257, Asp229, and Asp328. Glu257 is both a proton donor and acceptor, Asp229 forms a covalent intermediate with the cleaved substrate before CD formation, and Asp328 stabilizes the reaction intermediates [5, 110]. The detailed catalytic mechanisms of CGTases have been described by Van der Veen et al. [6], who suggested that the active site of most CGTases has approximately nine sugar-binding sites labeled as -7 to +2 [5, 14]. The +1 and +2 subsites stabilize the glucose ring with phenyl rings, whereas the -1 subsite constitutes the catalytic center. Residues at the -2 and -3 subsites exert significant effects on all four CGTase reactions, cyclization, coupling, disproportionation, and hydrolysis [7, 111]. The -6 and -7 subsites are involved in substrate binding [112]. The role of -4 and -5 subsites in CGTase activity has been reported in a few studies. Molecular modeling studies have indicated that even after the absence of strong interactions at the -4/-5 subsites, the substrates that are bare enough to form CDs will be favorably selected [113]. Tyrosine (Y195) is the predominant central residue in the active site of CGTases and is responsible for substrate specificity. Xie et al. [96] replaced this tyrosine residue with isoleucine in the CGTase of *Bacillus* sp. 602, resulting in a more flexible central site and shifting of product specificity from α-CDs to more β-CDs and γ-CDs. Moreover, structural

superimposition of mutant CGTase (Y195I) revealed that the residues at Lys232, Lys89, and Arg177 located at +2, -3, and -7 subsites, respectively, in the active domain could result in a smaller substrate-binding cavity [96].

A sequence alignment of selected CGTases (one *α*-CGTase, two *β*-CGTases, and one *γ*-CGTase) was performed, revealing seven conserved amino acid residues, or conserved sequence regions, in all *α*-amylase family enzymes (Figure 1). The residues in these conserved regions are directly associated with reaction catalysis as active site residues, in substrate binding, and as calcium-binding ligands [2, 39]. The sequence alignment further indicated that the *γ*-CGTases have various sequence differences over *α*-CGTases and *β*-CGTases (Table 4). The region of *α*-CGTases and *β*-CGTases between residues 145–152 (numbering based *N. circulans* 251 CGTase) constituting six amino acids located at subsite -7 (loop structure at the onset of B-domain in the tertiary structure) is completely missing in *γ*-CGTases. Similarly, in subsite -3, residue 47 determines product specificity and can be Arg and Lys (in *β*-CGTases), Lys (in *α*-CGTases), and Thr (in *γ*-CGTases). Another position is loop 87–93 in subsite -3, which constitutes only four amino acid residues in *γ*-CGTases; however, a stretch of seven amino acids is present in *α*-CGTases and *β*-CGTases. The two Ca^{2+} binding sites (CBSI and CBSII) were highly conserved in each CGTase sequence with only two variations (Asn/Asp29 and Asp/His199), thereby suggesting their role in deciphering the CD specificity. The residue near CBSII (residue 35) may determine CD specificity, wherein a Thr is located in *β*-CGTases, and *α*-CGTase and *γ*-CGTase have Ala and Gln at this place, respectively. In subsite +2, Ala is present in the sequence of *γ*-CGTase at position 232, while lysine is present at this location in both *α*-CGTases and *β*-CGTases. Overall, the product specificity of a *γ*-CGTase enzyme is mainly demonstrated by the residues located at subsite -7 and subsite -3 (Table 4).

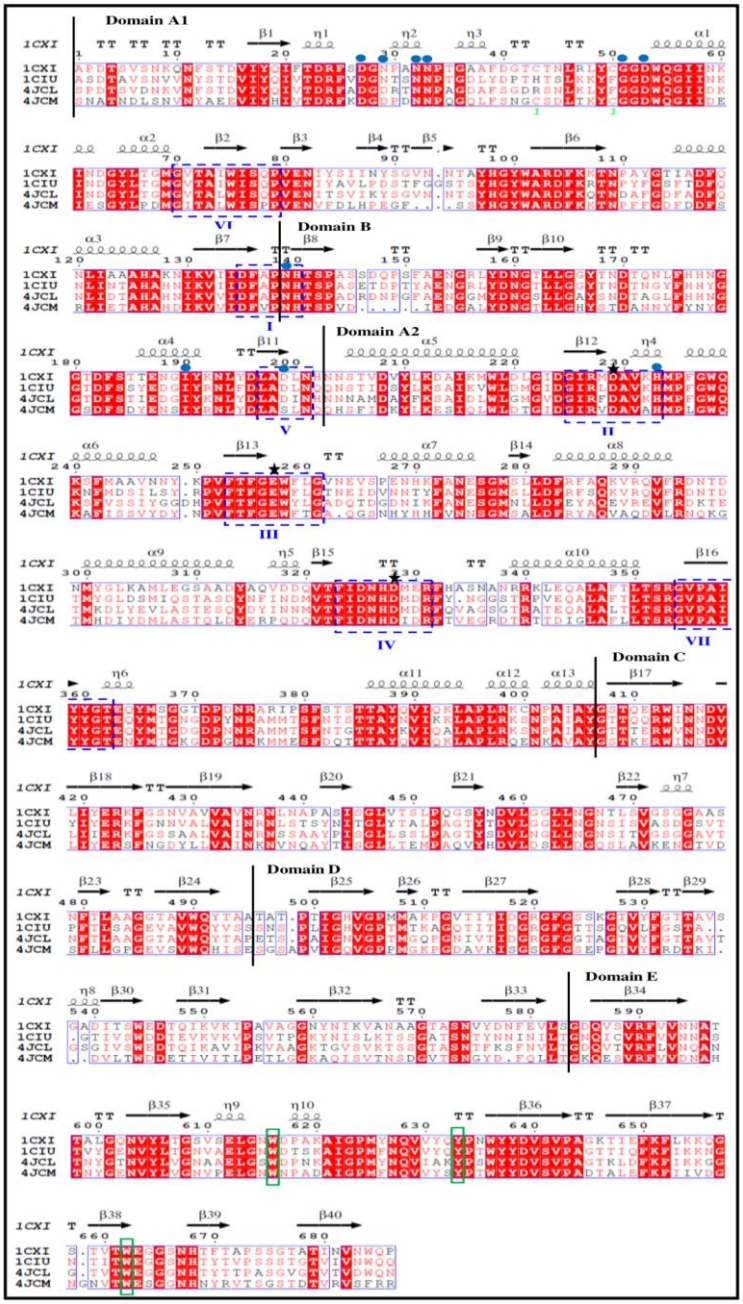

Figure 1 Multiple sequence alignment of selected CGTase sequences exhibiting sequence conservation 1CXI, 1CIU, 4JCL, and 4JCM represent PDB IDs of CGTases from *Niallia circulans* (*β*-CGTases), *Thermoanaerobacterium thermosulfurigenes* (*β*-CGTases), *Paenibacillus macerans* (*α*-CGTases), and *Evansella clarkii* (*γ*-CGTases), respectively. Sequence alignment was performed using the Multalign software, and structural alignment was performed using ESPript. The CGTase-specific domains (A to E) are marked individually by black lines, and the seven *α*-amylase family-specific regions (CSR-conserved sequence regions) are marked using dotted blue boxes. The calcium-binding sites (CBS) are indicated using blue dots over the sequence alignment as two distinct regions, CBSI (Asn139, Ile190, Asp/His199, and His233) and CBSII (Asp27, Asn/Asp29, Asn32, Asn33, Gly51, and Asp53). The catalytic site residues are represented by black stars, whereas maltose-binding sites are marked in green boxes. The regions in the sequences are marked according to a study by Liu et al. [62].

Table 4 Comparison of amino acid residues in different CGTases.

Location of residues		CGTase source			
Subsite	**Residue no.**	***N. circulans* (*β*-CGTase)**	***T. thermosulfurigenes* (*β*-CGTase)**	***P. macerans* (*α*-CGTase)**	***E. clarkii* (*γ*-CGTase)**
Central	**195**	Y	F	Y	Y
Near Ca^{2+} binding site	**32–36**	NNPTG	NNPTG	NNPAG	NNPQG
Subsite -7	**145–152**	SSDQPSFA	SETDPTYA	DRDNPGFA	D------I
	193	N	N	N	N
Subsite -6	**179–180**	GG	GG	GG	GG
	167	Y	Y	Y	Y
	47	R	K	K	T
Subsite -3	**196**	D	D	D	D
	371	D	D	D	D
	87–93	INYSGVN	LPDSTFG	IKYSGVN	HPEGF--
	375	R	R	R	R
Subsite -2	**101**	W	W	W	W
	98	H	H	H	H
	229	D	D	D	D
Subsite -1	**257**	E	E	E	E
	328	D	D	D	D
	233	H	H	H	H
Subsite +1	**230**	A	A	A	A
	194	L	L	L	L
	259	F	F	Y	F
Subsite +2	**232**	K	K	K	A
	183	F	F	F	F

Amino acid residues are numbered on the basis of *Niallia circulans* sequence, and the residues marked in red denote specificity for *γ*-CGTases only. PDB IDs for the sequences from *Niallia circulans*, *Thermoanaerobacterium thermosulfurigenes*, *Paenibacillus macerans*, and *Evansella clarkii* are 1CXI, 1CIU, 4JCL, and 4JCM, respectively.

6. Cyclization Mechanism in CGTases

CGTases follow an *α*-retaining double displacement mechanism similar to that of other *α*-amylase family enzymes [39, 110, 114, 115]. In brief, the mechanism is characterized by two catalytic residues, Asp229 (acts as a nucleophile) and Glu257 (an acid/base catalyst). The carboxylate group of Asp229 (C1) from the active site acts as a nucleophile to displace the leaving group, leading to the formation of an enzyme-substrate intermediate complex. The Asp229 is subsequently displaced by the acceptor group that is activated by the nonprotonated form of a general acid catalyst (Glu257/C2 carboxylate group) (Figure 2). The complete reaction mechanism can be explained in the following five steps:

1) Upon substrate binding, Glu257 donates a proton to the glycosidic bond oxygen atom; subsequently, the C1 of glucose present at subsite -1 faces a nucleophilic attack by Asp229.
2) A covalent intermediate is formed between Asp229 (enzyme) and the substrate after a transient oxocarbenium ion-like state detaches a linear oligosaccharide from the substrate molecule.
3) After protonation, the glucose molecule at subsite +1 exits the catalytic pocket (linear oligosaccharide/byproduct of cyclization reaction); subsequently, an acceptor glucose molecule attacks the covalent bond between Asp229 and the C1 of glucose at subsite -1.
4) A transient oxocarbenium ion-like intermediate is formed once again.
5) Glu257 acts as the base catalyst and takes a proton (H^+) from the incoming glucose molecule at subsite +1, allowing the oxygen atom of this glucose molecule to replace the oxocarbenium bond and leading to the formation of a new hydroxyl group at the C1 position of the new glycosidic bond.

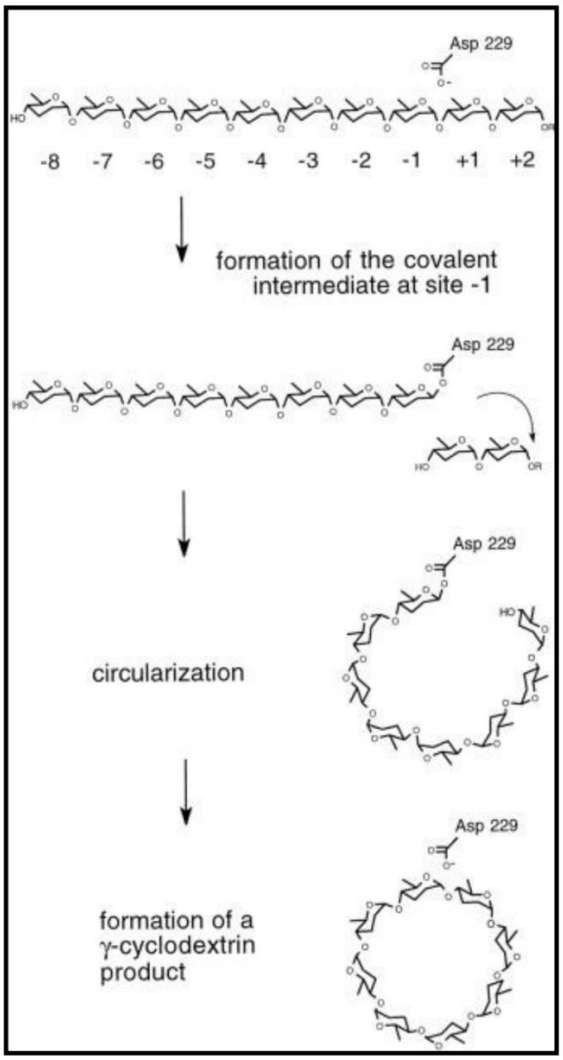

Figure 2 Schematic representation of cyclization reaction catalyzed by a *γ*-CGTase (adapted from Uitdeehag et al. [110] licensed under creative commons).

The reaction mechanism is similar for *α*-CGTases, *β*-CGTases, and *γ*-CGTases except for the distance between the acceptor glucose (step 3) and the glucose at subsite -1; this distance is 8, 9,

and 10 glucose residues for *α*-CGTases, *β*-CGTases, and *γ*-CGTases, respectively [39]. In most cases, this distance can be higher (several 100 glucose molecules), leading to the formation of large-ring CDs that are further converted to the size of *α*-CDs, *β*-CDs, and *γ*-CDs because of the coupling and hydrolytic activities of a CGTase [39, 115]. Moreover, the CGTase-catalyzed reaction is a reversible process, and CDs may convert further to a different product. The CDs produced can be cleaved to form linear CDs through a coupling reaction; the linear CDs can be cyclized again (in a cyclization reaction) to produce larger CDs than the original ones by various organic solvents/complexing agents (such as the conversion of *α*-CDs to *β*-CDs and *β*-CDs to *γ*-CDs). Furthermore, different mutagenesis approaches were adopted and reviewed in studies to enhance the product specificity of CGTases toward a particular CD; the approaches included site-directed mutagenesis, random mutagenesis, deletions or insertions, domain shuffling, and molecular imprinting [7, 14]. As mentioned previously in this review, the mutations at several subsites (such as subsite -3 and subsite -7) in the CGTase sequence can result in a significant change in product specificity, and few CGTases can produce *γ*-CDs specifically. Therefore, the mutations reported to enhance product specificity of a CGTase to *γ*-CD are compiled and presented in Table 5.

Table 5 Examples of site-directed mutagenesis improving *γ*-CD product specificity.

***CGTase* source**	**Mutation type**	**Subsite**	**Effect of mutation on product specificity**	**Reference**
E. clarkii	A223 and Gly255 replaced with acidic amino acids (Asp and Glu), basic amino acids (Lys, Arg, His), and serine	+2 (A223) and +3 (Gly255)	Presence of basic amino acids at position 223 improved *γ*-CD production without any loss in *β*-CD formation activity	Nakagawa et al. [116]
E. clarkii	A223K	+2	Usage of A223K CGTase together with isoamylase yielded 72.5% *γ*-CD	Wang et al. [12]
B. circulans 8	Deletion of six amino acids (145–151)	-7	Percentage of *γ*-CD production increased from 20% to 40%	Parsiegla et al. [117]
E. clarkii	Insertion of six amino acids (145–151) by using overlapping PCR		Production of *γ*-CD reduced from 76% to 12.5%	Yang et al. [118]
T. thermosulfurigenes	D371R	-3	*γ*-CD conversion increased from 4.9% to 7.5%	Wind et al. [92]
Bacillus sp. G1	H47T		Percentage of *γ*-CD production increased from 10% to 39%	Goh et al. [119]
E. clarkii	T47H, F91N/L/W, and Y186W	Subsite -3 and central	The *γ*-CD specificity (94.6%) of mutant Y186W improved than that of the	Wang et al. [17]

		residues	wild-type enzyme (77.1%)	
Paenibacillus sp. 602–1	Y195R and Y195I	Central residue	Product specificity shifted from *α*-CD to *γ*-CD and percentages of *γ*-CD production increased to 50% (Y195R) and 38% (Y195I)	Xie et al. [96]
Bacillus circulans 8	Y195W	Central residue	Percentage of *γ*-CD production increased from 20% to 50%	Parsiegla et al. [117]

7. Applications of CGTase

The CGTase is a multifunctional enzyme that catalyzes multiple enzymatic reactions, including cyclization, coupling, disproportionation, and hydrolysis reactions. Therefore, the CGTase enzyme finds applications in the synthesis of CDs (through cyclization) and several transglycosylation products (through coupling and disproportionation). In a recent report, CGTase from *Bacillus cereus* YUPP-10 (a cotton endophytic bacterium) was utilized as an antimicrobial protein that prevents the growth of *Verticillium dahlia* on cotton as a defensive response against verticillium wilt [107].

The transglycosylated products, namely glycosides, can also be synthesized using chemical transglycosylation; however, enzymatic transglycosylation is more favorable [5]. The enzyme-catalyzed transglycosylation offers various advantages over chemical transglycosylation, such as low steric hindrance, high regiospecificity, simpler reaction steps, mild reaction conditions, low production cost, and more eco-friendliness, apart from providing a characteristic anomeric configuration in a single step without the requirement for protection groups. Chemical transglycosylation requires higher reaction temperatures to perform acid catalysis in contrast to enzymatic transglycosylation. Moreover, chemical transglycosylation results in lower product yields and the formation of nonspecific anomers that are a mixture of *α*-anomers and *β*-anomers, thereby making the purification process more complicated. Apart from this, heavy metals are used as catalysts in chemical transglycosylation, thereby causing toxicity and increasing production costs. The nonspecific water-soluble compounds pose difficulties in the separation of desired glycoside compounds during the purification process [5].

Various enzymes, such as CGTase, pullulanase, dextranase, isomaltase, and *β*-galactosidase, can catalyze transglycosylation reactions. However, a CGTase is preferred over any other transglycosylase enzyme [5]. The disadvantages of other enzymes include lower yields, partial hydrolysis of glycoside compounds, poor regioselectivity, and the formation of various undesired compounds. By contrast, a CGTase provides good conversion yields for glycosylated products, low hydrolytic activity, a higher degree of transglycosylation for certain acceptors, and high regioselectivity, that is, specificity to catalyze *α*-(1→4)-glycosyl transfer reactions [5]. The transglycosylation reaction of various CGTase enzymes and their subsequent transglycosylated products have been comprehensively reviewed recently by Lim et al. [5] and, therefore, has not been discussed in the present review. The current review majorly focuses on the cyclization

reaction of CGTases for the synthesis of CDs and the application of resultant CDs in various fields. Before the discussion of CDs and their applications, enzyme immobilization is described in the following section because immobilization is a way of reducing the costs of enzyme-mediated catalytic processes.

8. CGTase Immobilization

CGTases (EC 2.4.1.19) are specialized enzymes that act on starch or related sugars and are currently used for the industrial production of CDs. The major bottleneck in enzyme-based industrial production is the high enzyme cost, which can be significantly reduced by improving the operational stability of an enzyme through its immobilization [5]; CGTase immobilization has been recently reviewed by several studies [120, 121]. Various matrices have been reported for CGTase immobilization, such as agar, glyoxyl-agarose, alginate, chitin, chitosan, carrageenan, cross-linking on PVA nanofibres, silica nanospheres, magnetic carriers, eupergit C, and CLEA; electrostatic interaction-based immobilization on pineapple peal and surface immobilization have also been performed. Some of the matrices used for CGTase immobilization are tabulated in Table 6. Of several procedures for enzyme immobilization, including adsorption, cross-linking, covalent binding, and entrapment [122], covalent cross-linking of the enzyme is highly desired because the cross-linked enzyme exhibits more stability and does not leach out.

Table 6 CGTase immobilization on various support matrices.

Immobilization Type	CGTase Source	CD Type	Activity Recovery (%)	Recyclability	Remark	Reference
Fe_3O_4 nanoparticles activated with polydopamine	NR	*β*-CD	78	Retained 19% activity after nine cycles	Immobilized enzyme was optimally active at pH 6 and a temperature of 55 °C and 50 °C for *β*-CGTase and *γ*-CGTase, respectively. Catalytic efficiency of immobilized enzyme was around 3.1 and 2.2-fold higher for *β*-CGTase and *γ*-CGTase, respectively	Zhang et al. [123]
Cellulose nanofibers (CNFs) coupled with 1,12-diaminododecane	*Bacillus macerans* (commercial CGTase from Amano enzyme Inc., Japan)	*α*-CD	69	Retained 68% activity after 10 cycles	A change in thermal stability was observed from 60 °C to 70 °C	Sulaiman et al. [124]
Silica coated-magnetic nanoparticles functionalized with APTES	*Amphibacillus* sp. NPST-10	NR	73	Retained 67% activity after 10 cycles	After CGTase immobilization, pH optima reduced, whereas temperature optima, thermostability, and substrate affinity increased	Ibrahim et al. [125]

Polyvinyl alcohol (PVA) nanofibers prepared through electrospinning and cross-linking with glutaraldehyde	*Bacillus macerans* (commercial CGTase from Amano enzyme Inc., Japan)	NR	95	NR	Immobilized CGTase exhibited 2.5-fold higher enzyme loading capacity and 31% higher enzyme activity	Saallah et al. [126]
Epoxy-activated acrylic beads with different pore sizes (Eupergit C and Eupergit C 250L)	*Thermoanaerobacter* sp.	Mixture of *α*-CD and *β*-CD (31%) with oligosaccharide formation (69%)	10.2 (Eupergit C) and 4.8 (Eupergit C 250 L)	Eupergit C immobilized enzyme retained 40% activity after 10 cycles of 24 h each	Half-life of Eupergit C immobilized CGTase at 95 °C enhanced five times	Martin et al. [127]
Silica preactivated with glutaraldehyde functionalized with APTMS	*Thermoanaerobacter* sp. (commercial CGTase: Toruzyme from Novozymes A/S, Denmark)	Mixture of *α*-CD, *β*-CD, and *γ*-CD	3–5	NR	Longer reaction time and pH < 5 favored *γ*-CD formation, whereas *α*-CD, and *β*-CD formation was favored in reverse conditions	da Natividade Schoffer et al. [128]
Covalent attachment on Glyoxyl-agarose	*Thermoanaerobacter* sp. (commercial CGTase: Toruzyme from Novozymes A/S, Denmark)	*β*-CD	32	NR	*β*-CD yield with immobilized CGTase was two times higher than free enzyme	Tardioli et al. [129]
Sulfopropyl-sepharose	*Bacillus macerans*	*α*-CD	95	10% decrease in productivity after 10 days	CD production was performed in a packed bed reactor	Kweon et al. [130]
Silica, chitosan, and	*Bacillus firmus* strain	*β*-CD	<5	NR	Highest immobilization	Sobral et

alumina	no. 37				recovery was achieved on silica with HEMDA at 7 °C without stirring and 26 °C with stirring	al. [131]
Chitosan preactivated with glutaraldehyde	*Thermoanaerobacter* sp. (commercial CGTase: Toruzyme from Novozymes A/S, Denmark)	*β*-CD	6	60% activity was retained after 100 batches of 15 min. in a packed bed-reactor	Maximum productivity of 310 g *β*-CD/L h was achieved at 4% substrate concentration and a flow rate of 5 mL/min	da Natividade Schoffer et al. [132]
Covalent cross-linking on chitin	*Bacillus agaradhaerens* KSU-A11	NR	85	Retained almost 50% initial activity after nine cycles; *β*-CGTase activity was reduced to 30% after six batch cycles, and *γ*-CGTase activity was reduced to 0% after six batch cycles	Immobilized CGTase was more thermostable and has higher Km value	Ibrahim et al. [133]
CLEA	*Bacillus pseudalcaliphilus* 8SB	*β*-CD	8.5		Free enzyme conformation was maintained even at 85 °C; *β*-CGTase activity in CLEA was 20 times higher than *γ*-CGTase activity	Zhang et al. [134]
	Thermoanaerobacter sp. (commercial CGTase: Toruzyme from Novozymes A/S, Denmark)	Mixture of *α*-CD, *β*-CD, and *γ*-CD	24	Reused till five cycles of 3 h each, with 80% of initial productivity retainment	Use of prehydrolyzed starch by *α*-amylase resulted in a 45% CD yield	Rojas et al. [135]
Gelatin	*Bacillus circulans* STB01	*β*-CD	23.1	Retained 92% initial activity after 20 cycles of enzyme activity	A broad pH optimum with two peaks (at pH 5.5 and pH 8.5) together with a higher pH and	Chen et al. [136]

					thermostability was observed after immobilization	
Cell entrapment in calcium alginate, K-carrageenan, agar-agar, and gelatin	*Bacillus* sp. TPR71HNA6	NR	NR	Successful CGTase production was achieved for seven batches (calcium alginate and agar-agar), five batches (gelatin), and four batches (carrageenan)	Calcium alginate was best as a support matrix for CGTase production	Ravinder et al. [137]
Electrostatic interaction-based immobilization on pineapple peal	*Bacillus licheniformis*	*α*-CD	76	NR	NR	Man et al. [138]
Bacterial cell immobilization on agar	*Bacillus circulans* ATCC 21783	*β*-CD	NR	~100% initial activity was observed after five cycles of 48 h each in a fluidized bed reactor	180–210 U/mL of enzyme activity achieved after 24 and 48 h cultivation with entrapped bacterial cells	Vassileva et al. [139]
Cell surface of *Saccharomyces cerevisiae*	Recombinant CGTase from *Bacillus circulans* 251	*α*-CDs, *β*-CDs, and *γ*-CDs with glucose and maltose	NR	NR	Yeast cells with CGTase exhibited on surface produced 24.07 mg/mL CD in yeast medium	Wang et al. [51]

NR indicates that the required data is not reported in the cited paper.

9. CDs: Structure, History, Enzymatic Synthesis, and Applications

CDs are cyclic oligosaccharides linked by α-1,4-glycosidic bonds and are classified according to the number of glucose units present. The three major types of CDs are α-CDs, β-CDs, and γ-CDs, which carry six, seven, and eight glucose molecules, respectively [9]. These CDs are geometrically arranged as hollow truncated cones, and their structure and applications have been extensively studied. The CD structure has an external more hydrophilic side and an inner less-hydrophilic pocket that provide space to accommodate different hydrophobic molecules in solutions. Therefore, CD finds wide applications in several industrial sectors, such as food, pharmaceuticals, plastics, cosmetics, environment, and agrochemical industries [2, 11, 12].

9.1 CD Structure

In CDs, the glucose units are arranged in a circular fashion, resulting in a frustum-like shape (a hollow truncated cone). The inner cavity is less hydrophilic than the outer surface due to the presence of hydrogen atoms and glycosidic bonds having oxygen atoms. The outer surface is more hydrophilic due to the presence of free hydroxyl groups that facilitates the formation of inclusion complexes with various hydrophobic compounds [140]. CD structure has two faces, primary and secondary faces (Figure 3a). The primary hydroxyl groups (-CH_2OH) constitute the narrow edge or primary face, whereas secondary hydroxyls (-CHOH) form the wider edge or secondary face [141].

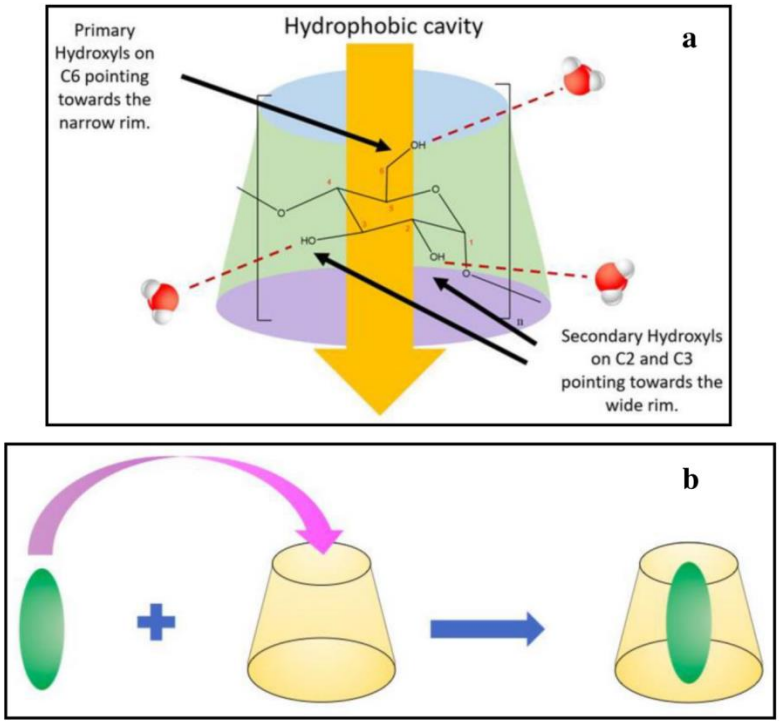

Figure 3 Structure of CD and formation of inclusion complex: **a** Model representing the toroidal structure of CD with its primary and secondary faces; **b** General mechanism of CD-guest inclusion complex formation (adapted from Poulson et al. [10] licensed under creative commons).

Among *α*-CDs, *β*-CDs, and *γ*-CDs, *γ*-CDs have a bigger cavity size due to a greater number of glucose units. The CD cavity size is related to its solubility in solution and also assists in the formation of inclusion complexes (Figure 3b) with compounds that face problems related to bioavailability, stability, and water solubility [39]. Stoichiometry of some CD inclusion complexes is affected by the CD type and guest molecule. Various techniques, such as NMR, X-ray diffraction, and differential scanning colorimetry, together with a classical phase solubility analysis method, are used to evaluate the stability of the CD complex [142].

9.2 CD Types

Because CD structure comprises cyclic rings arranged circularly, CDs are majorly classified into *α*-(6), *β*-(7), and *γ*-CDs (8), respectively, according to the number of glucose residues (Figure 4); they exhibit several properties [10, 143] that are tabulated in Table 7. In a CGTase-catalyzed conversion process, CDs with less than six glucose units are unstable due to steric hindrance, whereas CDs with more than nine glucose units cannot be easily purified [144]. The CD containing 32 glucose units is the largest known well-characterized large ring cyclodextrin (LR-CD); however, LR-CDs with up to 150 glucose residues are also known [145, 146]. Because of the bigger cavity of *γ*-CD than *α*-CD and *β*-CD, it can carry large-sized molecules inside the cavity to form inclusion complexes. The formation of an H-bond between hydroxyl groups attached to C2 and C3 carbon atoms of adjacent glucose residues contributes to CD stability by stabilizing the crystal lattice [147].

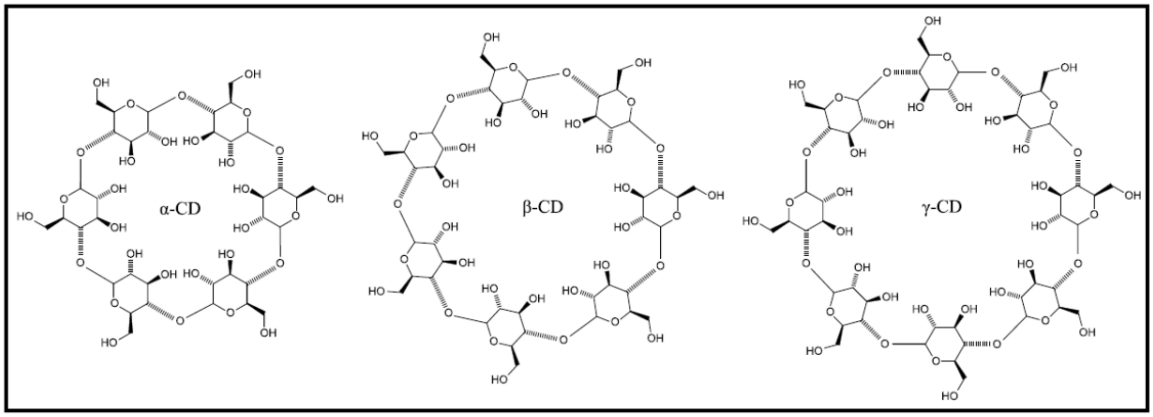

Figure 4 Chemical structure of *α*-CDs, *β*-CDs, and *γ*-CDs (adapted from Wikipedia licensed under creative commons).

Table 7 Types of CDs and their properties.

Properties	*α*-CD	*β*-CD	*γ*-CD
No. of glucopyranose units	6	7	8
Molecular weight (g/mol)	972.9	1135	1297.1
Central cavity diameter (Å)	4.7–5.3	6.0–6.5	7.5–8.3
Solubility in water at 25 °C (g/L)	145	18.5	232
pKa	12.3	12.2	12.1
No. of water molecules in cavity	6–8	11–12	13–17

Of the three major CDs, *β*-CD has the lowest water solubility because of the rigidity of its molecular structure and the effect caused by the intermolecular hydrogen bonding between neighboring C2-OH and C3-OH in the crystal state, which counteracts its hydration with surrounding the water molecules [148]. However, an incomplete belt of such hydrogen bonds in *α*-CD is noted, and the structure of *γ*-CD is noncoplanar. Therefore, both *α*-CD and *γ*-CD have higher water solubility than *β*-CD [10, 148]. *β*-CD derivatives produced by the substitution of the-OH groups result in the disruption of these H-bonds and lead to an increase in water solubility; various such derivatives are available in the market [140, 147, 149] and some of these common CD derivatives are listed in Table 8.

Table 8 Common CD derivatives with higher water solubility.

CD	Molecular Weight (g/mol)	Average No. of Substitution Per Glucopyranose Unit	Solubility in Water (mg/mL) at 25 °C
HP (hydroxypropyl)-*β*-CD	1400	0.65	>600
HP-*γ*-CD	1570	0.60	>500
β-CD SBE (sulfobutylether) sodium salt	2160	0.90	>500
Heptakis (2,6-di-O-methyl)-*β*-CD (DIMEB)	1331	2	>500
Heptakis-(2,3,6-tri-O-methyl)-*β*-CD (TRIMEB)	1429	3	290
Randomly methylated *β*-CD (RAMEB)	1310	1.80	>500

9.3 Historical Background: A Comprehensive Timeline

CDs are crystalline substances that were first obtained from the bacterial digest of starch by Antoine Villiers in 1891 [150]. He named this crystalline substance “cellulosine” because of its cellulosic properties, such as resistance to acid hydrolysis and a lack of reducing properties. After the evaluation of these crystallized dextrins for some years, Schardinger fractionated *α*-dextrins and *β*-dextrins in 1903, which are now known as *α*-CDs and *β*-CDs [151]. Till 1911, he conducted several studies on cellulosine’s properties; therefore, he is considered the “Founding father of Cyclodextrins.” A s a tribute to him, cellulosines were subsequently named Schardinger dextrins [152, 153]. In 1935, Freudenberg et al. discovered the current *γ*-CDs [154]. In the late 1930s, Freudenberg et al. discovered that these Schardinger dextrins have a cyclic structure and are made of maltose units with an *α*-1,4 glycosidic bond [155, 156]. In the early 1940s, Schardinger dextrins were renamed “cycloamylases” by Dexter French, an American chemist, and finally “cyclodextrins” (CDs) in the late 1940s by Friedrich Cramer, a German chemist [157]. In the same period (around the 1950s), Friedrich Cramer and French with their coworkers evaluated the enzymatic production of CDs and their purification methods. Subsequently, French published the first review article on CDs [158-160]. Around the same time, French and Pulley discovered that large cavity CDs with 9, 10, and 11 glucose units also exist [142]. In 1981, the first international

convocation was organized on CDs [14]. Since then, a lot of research has been conducted because of their importance in various fields, such as pharmaceuticals and drug delivery. A detailed historical examination of CDs has been presented in several reviews [152, 157, 159, 161]; however, a chronological advancement in CGTases and CDs has only been presented here (Figure 5).

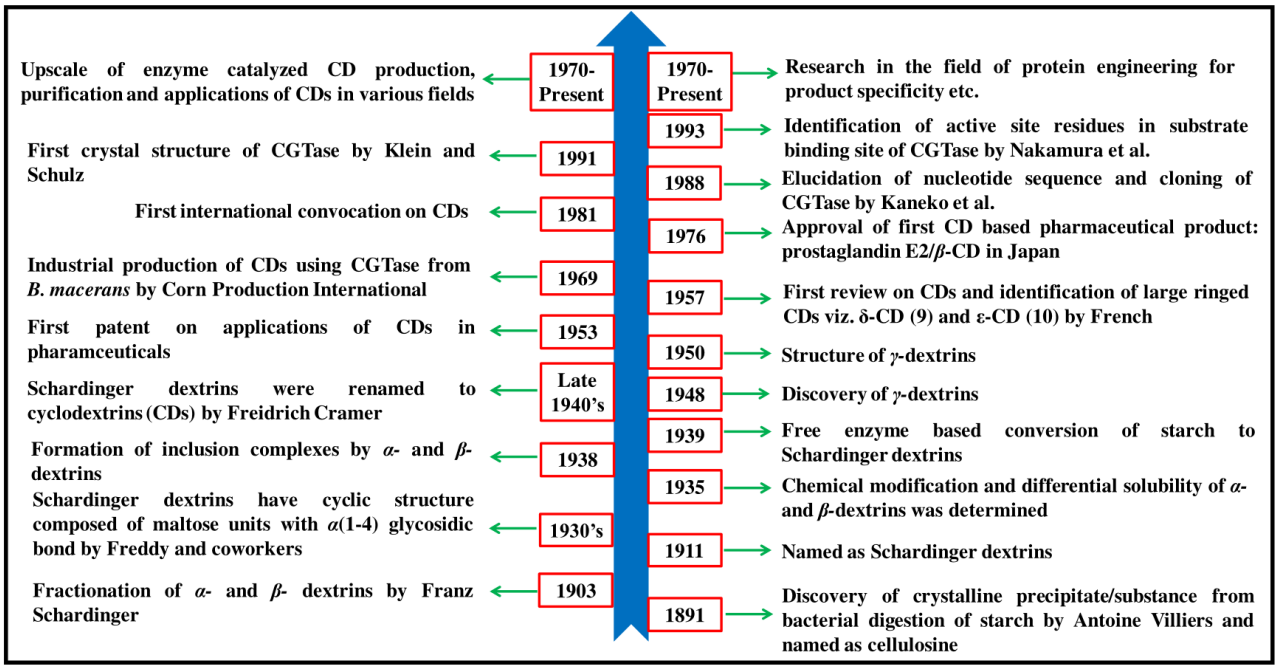

Figure 5 Milestone discoveries in the field of CGTases and CDs.

9.4 Enzymatic Synthesis of CDs

The CD production using microbial CGTases is well-studied. However, all known CGTases produce a mixture of CDs with higher ratios of *β*-CDs, followed by *α*-CDs and *γ*-CDs together with large-ring CDs [16, 17, 162]. Of these, *γ*-CDs are more desirable because of their unique properties over *α*-CDs and *β*-CDs. They have a larger internal cavity and a noncoplanar and a more flexible structure, which gives them much higher solubility than *α*-CDs and *β*-CDs [2, 163]. However, *γ*-CDs are less available in the market than *α*-CDs and *β*-CDs. Approximately 70% of the total CD production globally comprises *β*-CDs, followed by *α*-CDs (15%); *γ*-CD production is only 5% of the total CD production [2, 163, 164]. Till now, CGTases from *E. clarkii* [25], *Bacillus* sp. G-825–6 [16, 28], and *B. thuringiensis* GU-2 [37] produce *γ*-CDs as the major product. Therefore, CGTase enzymes capable of producing an increased ratio of *γ*-CD are desirable to fulfill the increasing demand. In addition, the end product inhibition and coupling activity of CGTases are hurdles that limit final CD yields [2, 14, 165]. Therefore, numerous ways are being sought to overcome these limitations in CD production together with product separation processes, such as continuous removal by using the membrane filtration process, ion-exchange chromatography, and affinity chromatography [2, 165, 166].

Various parameters determine the amount and type of CD formation, such as the nature of substrate, source of CGTase, type of complexing agent, and optimization of reaction conditions [15]. In addition, yields and selectivity of CD production can be altered through the molecular engineering of existing CGTases [7], the use of immobilized enzymes, and the identification of

novel CGTases. In a study, the optimization of reaction parameters for CD conversion produced nearly 7 mg/mL *γ*-CD from 78 mU/g soluble starch at pH 10 [28]. Alves-Prado et al. [167] achieved approximately 80% starch conversion to CDs (a mixture of *α*-CDs, *β*-CDs, and *γ*-CDs) by using 1% soluble starch when screening various starch sources for conversion. Furthermore, Wu et al. [15] optimized various enzymatic conditions and reported a 30% conversion of starch to CDs at pH 12 and 60 °C by using 5 U/g of soluble starch in a nonsolvent process. The conversion was thereafter enhanced to 57% by the addition of 2% glycyrrhizic acid in a solvent process. In another study, stepwise optimization of reaction parameters was performed to obtain 50.4% CD yield from 15% potato starch and by using *γ*-CGTase from *E. clarkii* with the addition of cyclododecanone (a solvent process) [12]. In subsequent studies, the CD yields increased to 72.6% following optimized conditions in the presence of cyclododecanone and by using an enzyme variant Y186W for *γ*-CD production [17]. Large-size CD production can also be influenced by minor reactions of CGTases, such as coupling and hydrolysis. In such cases, the production of large-sized CDs can be significantly increased just by the adjustments of incubation time and temperature [168, 169].

Site-directed mutagenesis was performed in the subsite +2 at the Ala223 and Gly255 residues in the *γ*-CGTase sequence from *E. clarkii* [116]. The *γ*-CGTase activity increased, leading to higher *γ*-CD yields when Ala223 was substituted with basic amino acids (lysine, arginine, and histidine); however, the substitution of Gly255 with these basic residues resulted in reduced *γ*-CD yields. Tardioli et al. [170] used immobilized CGTases for enhancing the production of *β*-CDs. In addition, pretreatment of starch with pullulanase (a debranching enzyme) increased the CD yield by 4% to 6% [171].

9.5 Enzyme-Based CD Production Processes

On the basis of the requirement of a complexing agent, two general processes, namely a solvent process and a nonsolvent CD separation process, are employed for CD production (Figure 6). Both processes involve five major steps and differ in their production and extraction steps. Moreover, the CGTase is inhibited by the formed product in both processes. Therefore, to achieve high conversion yields (starch to CDs), the product should be separated from the mixture as it is produced. A recently developed process [172] achieved this through a liquid biphasic separation process, wherein the produced CD and CGTase are separated in different phases, and the biocatalyst is continuously recycled during the production and separation of CDs.

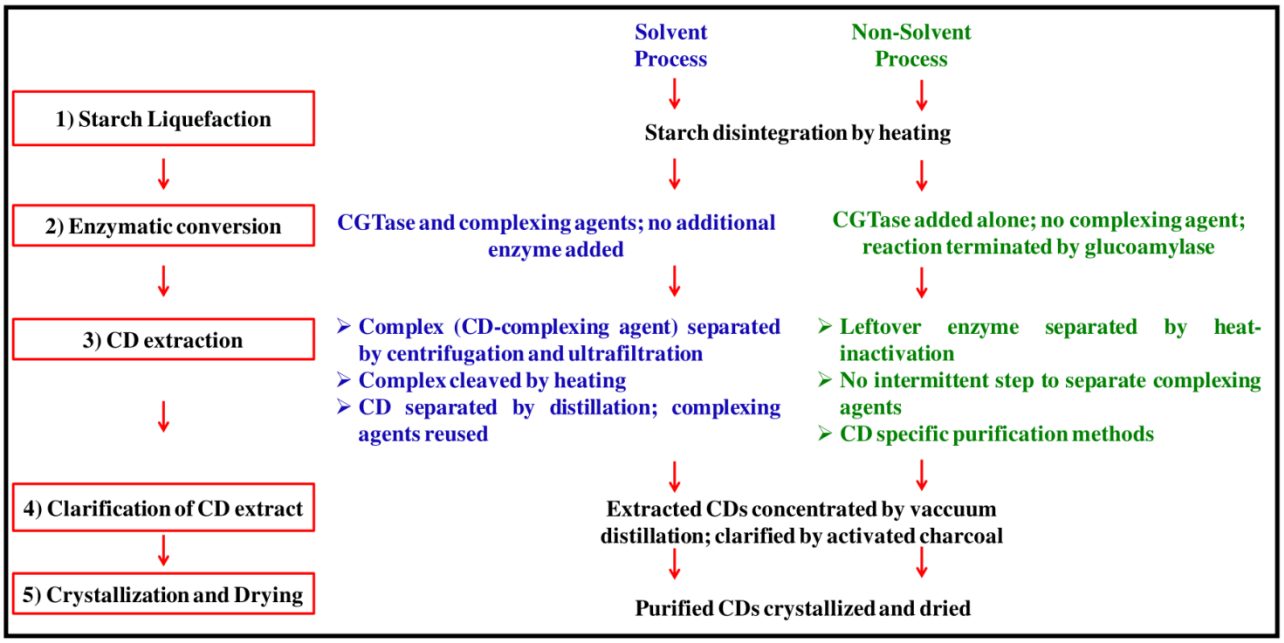

Figure 6 Schematic representation of the solvent and nonsolvent processes for CD production.

9.5.1 Solvent Process

The solvent process is the most common method in the industrial production of CDs, and the method uses a complexing agent to extract the CD from the solution [2]. First, liquefaction of starch is performed using mechanical disintegration or either heat-stable *α*-amylase. As the reaction proceeds, a complexing agent is added according to the CD to be isolated [165]. For example, n-decanol, cyclohexane, and acetonitrile are specific for *α*-CDs; toluene for *β*-CDs, and bromobenzene and glycyrrhizic acid for *γ*-CDs. The CD-complexing agent complex reduces product inhibition that occurs due to the accumulation of CDs in the reaction mixture. After the completion of the reaction, centrifugation or ultrafiltration is performed to separate the complex from the impurities. The remaining solution contains components, such as left-out starch, complexing agent, glucose, and maltose. Excess complexing agents are removed through washing and thereby reused. Subsequently, heating is performed to cleave the complex, and the complexing agent is separated from the CD by using a distillation process [165]. Few complexing agents are difficult to remove through the distillation process; therefore, the liquid-liquid extraction method is used for such agents [173]. The obtained product is concentrated through vacuum distillation, and crystallization is performed to obtain crystallized CDs that are then washed and dried. These downstream steps in the solvent process are not particular to CDs and only remove other impurities from CDs [165]. Thus, the type of complexing agent and CGTase determine the type of CD produced [174].

9.5.2 Nonsolvent Process

The nonsolvent process does not involve the use of any complexing agents or organic solvents for CD extraction and is, therefore, preferred in food industries where the use of solvents is discouraged. This process has also been used on an industrial scale for commercial CD

preparations [165]. First, the liquefaction of starch is performed, followed by an enzymatic reaction similar to the solvent process; however, reaction termination is achieved after the addition of glucoamylase, which converts leftover dextrins and malto-oligosaccharides to maltose and glucose. Subsequently, CGTase is either inactivated through heating or separated if an immobilized enzyme is used. The final products, i.e., glucose and maltose, do not interfere with the purification steps of CDs. The reaction mixture is then clarified using activated charcoal treatment, followed by concentration under reduced pressure (vacuum) and crystallization and recrystallization strategies. Although this process results in lower yields, it has the advantage of being completely solvent-free [165].

9.5.3 Liquid Biphasic System for Continuous Synthesis and Separation of CDs

Aqueous two-phase partitioning has also been used for the separation of CGTase and CD in two different phases. It is an extractive bioconversion process, wherein the production and extraction of CDs are achieved in a single pot [175]. The process is advantageous as the biocatalyst is recovered for continuous bioconversion together with the product [172, 175]. However, the aqueous phase partitioning system is still not adopted on a commercial scale due to its cost and complex partitioning mechanism [176]. As mentioned previously, *γ*-CDs are more costly than *α*-CDs and *β*-CDs due to their lower production rates. Moreover, they are highly desirable in food and pharmaceutical industries over *α*-CDs and *β*-CDs because of their high aqueous solubility, biodegradability, and large internal cavity that enables the formation of inclusion complexes with comparatively larger organic molecules in large concentrations [2]. In this respect, the use of cost-effective polymers, such as EOPO (ethylene oxide-propylene oxide), as copolymers [172] other than conventional PEG [177] has been reported for the production of *γ*-CDs by using *B. cereus* CGTase.

EOPO is a thermos-separating polymer that breaks and separates into two phases as a function of temperature [178]. In extractive bioconversion, EOPO has been used for the recovery of *γ*-CDs, with the recovery of *γ*-CDs being the function of EOPO concentration. A recovery of 17.5% *γ*-CD was achieved after 2 h of the recovery process [172]. A repetitive batch study indicated that with the repeated extraction of CDs from the top phase, enzyme activity continuously declined as EOPO also got extracted together with CDs. Therefore, the enzyme from the bottom phase should not be recycled more than once to have effective bioconversion yields [172].

9.5.4 Other Methods for CD Separation

For the purification of CDs from the rest of the reaction mixture and for separating a particular CD type, various methods are employed, including affinity chromatography, thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and membrane filtration processes. Affinity chromatography is a type of liquid chromatography and is based on a specific interaction between an immobilized ligand on a column and its binding partner. For example, 1,8-naphthylic acid anhydride bound to an aminated column (Biogel P-6) specifically binds to *γ*-CDs and is completely separated from a mixture of other cyclic and acyclic dextrins [179]. TLC is a basic technique used to separate analytes in the mixture and is based on their relative affinity with the mobile and solid phases. It can be used for the detection and approximate quantification of CDs present in the mixture [38]. In HPLC, separation is performed on the basis of the analyte

distribution in the mixture between the stationary phase and liquid phase. It is the most commonly used and effective method for CD separation and quantification [12, 16, 25, 28]. Membrane filtration or ultrafiltration is the conventional method of CD purification. The membrane enables CD to pass through it but stops the passage of enzymes and other undesirable starch degradation products [165, 166, 180].

9.6 Applications of CDs

Because of the wide range of applications, several studies have focused on CDs in the last decade [5, 153, 181]. A crucial property of CD is its ability to form inclusion complexes with a wide range of molecules. The formation of an inclusion complex does not involve the breakage of covalent bonds but is driven by Van der Waals interaction, H-bonding, and the removal of energy-rich water from the cavity [153]. Another characteristic feature of natural CDs is their ability to produce different CD derivatives through the substitution of the-OH groups; the derivatives can be further categorized as nonionic, cationic, and anionic CD derivatives. Some examples of nonionic CD derivatives include (2-hydroxy) propyl-*β*-CD (HP-*β*-CD) and randomly methylated-*β*-CD (RM-*β*-CD); cationic CD derivatives include permethylated propylene diamine-*β*-CD (PEMPDA-*β*-CD), heptakis (6-deoxy-6-amino)-*β*-CDs (HA-*β*-CD), and permethylated 6-monoamino-6-monodeoxy-*β*-CD (PMMA-*β*-CD); and sulfobutylether-*β*-CD (SBE-*β*-CD) and sugammadex (Bridion) are anionic CD derivatives.

Although natural CDs and their complexes are hydrophilic, their aqueous solubility is limited, especially for *β*-CD. This is because of the strong binding of CD molecules in their crystal state [182]. CDs contain glucopyranose units that further consist of three free hydroxyl groups differing in both function and reactivity. Different reaction conditions, such as pH, temperature, and reagents, affect the relative reactivity of secondary hydroxyls (C2-OH and C3-OH) and primary hydroxyls (C6-OH). These hydroxyls are formed by the substitution of -OH and -H groups in CDs with groups, such as hydroxyl alkyl, amino, carboxy alkyl, alkyl, and glucosyl. The CD derivatives thus formed have increased solubility and complex forming ability, which helps in CD polymer formation [183, 184], thereby increasing its applicability in various fields (Figure 7).

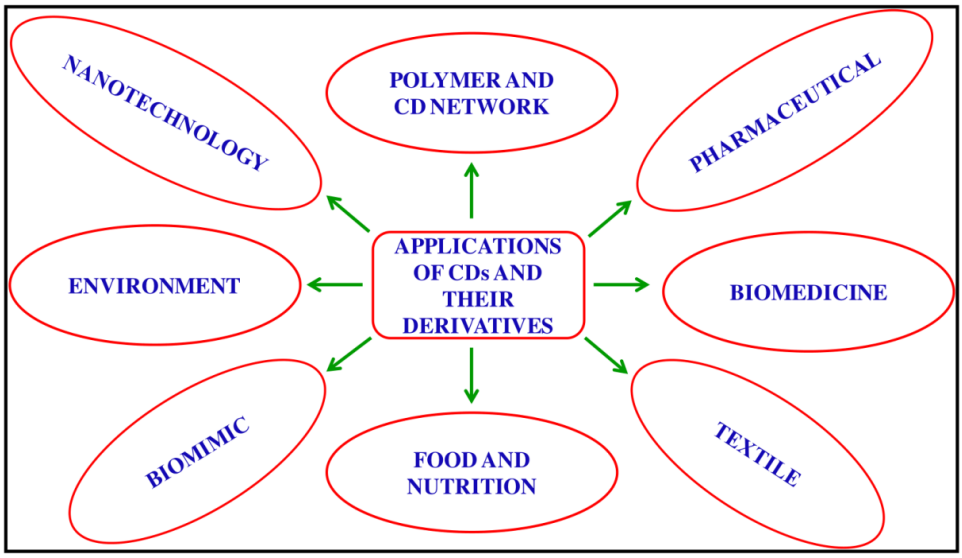

Figure 7 Applications of CDs and their derivatives in various fields.

9.6.1 Role of CDs in Pharmaceuticals

CDs and their derivatives have various applications in the pharmaceutical sector [185, 186, 187] as they provide benefits, such as improving the efficacy of existing drugs and their bioavailability. The efficacy of various drugs is improved by increasing their stability [181]. For example, CME-*β*-CD (O-carboxymethyl-O-ethyl-beta-CD), a CD derivative, enhances the stability of PG-E (prostaglandin E). Currently, more than 50 CD-containing medication products are available on the market [188]. On the basis of the inclusion of complex formed and reaction conditions, CDs slow or fasten various reactions, such as hydrolysis, isomerization, and oxidation reactions [181]. Because of their safety and solubility characteristics, together with their ability to form inclusion complexes, CDs improve the bioavailability of drugs. In biosafety studies, CDs reduced the toxic side effects of various drugs that arise due to low solubility and are, therefore, beneficial in delivering lipophilic drugs. CDs assist in controlled drug release, which can be either pH-controlled, osmotically controlled, or dissolution-controlled [181, 185]. Amphiphilic CDs are widely used as nanoparticles in drug delivery [141]. Applications of CDs in this field have been extensively reviewed, and some of the recent reviews are tabulated below (Table 9).

Table 9 List of reviews (2020 to present) on the role of CDs in the field of drug delivery.

Title of Review	**Focus of Review**	**Reference**
The Lord of the NanoRings: Cyclodextrins and the Battle Against SARS-CoV-2	Various roles of CDs as rings against SARS-CoV-2. For example, the encapsulation and transportation of specific drugs, such as vaccines, cholesterol trappers, and antiviral compounds.	Garrido et al. [188]
Cyclodextrins in Antiviral Therapeutics and Vaccines	Role of CDs in solubilization and stabilization of antiviral drugs together with its usage in antiviral vaccine formulations as mRNA delivery systems and as APIs (active pharmaceutical ingredients) against viral diseases	Braga et al. [189]
Cyclodextrins in Antiviral Therapy	Summary of the basic properties of CDs and their derivatives together with their role in antiviral formulations for the prevention and treatment of viral infections. The review also discusses new biologically active CD derivatives.	Jicsinszky et al. [190]
Versatile Nasal Application of Cyclodextrins: Excipients and/or Actives?	Utilization of CDs in nasal formulations in the past two decades, wherein CDs act as APIs, excipients, and absorption promoters	Rassu et al. [191]
Cyclic Oligosaccharides as Active Drugs, an Updated Review	Role of CDs in various fields, such as active drugs against various diseases, in dietary roles, and for cell differentiation.	Matencio et al. [192]
Cyclodextrin-based Pickering Emulsions: Functional Properties and Drug Delivery	Focusses on methods to prepare Pickering emulsions and lipid encapsulation together with features of CD-based Pickering emulsions and	Jug et al. [193]

Applications	their applications in cosmetics, drug delivery, as antifungals, and future perspectives.	
Cyclodextrins in Drug Delivery: Applications in Gene and Combination Therapy	Role of CDs in gene therapy together with their utilization in combination therapy against diseases, such as cancer and various genetic disorders.	Haley et al. [194]
Current Trends of Targeted Drug Delivery for Oral Cancer Therapy	Recent developments in targeted oral drug delivery against oral cancer and various delivery systems, including CDs.	Zhang et al. [195]
Biocompatible Polymers Combined with Cyclodextrins: Fascinating Materials for Drug Delivery Applications	Design of nano and micro carriers, fibers based on polymer or CD supramolecular systems, and hydrogels together with their applications as drug delivery systems.	Kost et al. [196]
Cyclodextrins-Peptide/Protein Conjugates: Synthesis, Properties, and Applications	Various ways to synthesize CD conjugates and their role in drug delivery against various diseases.	Lagiewka et al. [197]
Cyclodextrin-Containing Hydrogels: A Review of Preparation Method, Drug Delivery, and Degradation Behavior	Various methods for the preparation of CDs containing hydrogels and their utilization in the biomedical sector.	Liu et al. [198]
Cyclodextrin-Based Nanoparticles for Drug Delivery and Theranostics	Application of CDs as nanosystems for efficient drug delivery and their usage in diagnosis and therapeutics of various diseases.	Gadade and Pekamwar [199]

9.6.2 Polymers and CD Network

CD and its derivatives are widely used to make CD polymers with enormous applications. CD-based polymeric materials, such as nano or microparticles, hydrogels, organic resins, and other cross-linked materials, are frequently used in areas, such as water purification, separation processes, remediation, tissue engineering, medical diagnostics, and pharmaceuticals [200]. For the formation of CD polymers, covalent cross-linking is required between CDs or between CDs and a polymer by using a crosslinker, such as epichlorohydrin, dibasic acid dichlorides, and diisocyanates [198, 201, 202]. For example, epichlorohydrin is commonly used to cross-link *β*-CD [198, 201].

CD polymers are of three types, water-soluble, water-insoluble, and immobilized CD polymer. Water-soluble CD polymers have a low ratio of CD monomers, and an increase in CD monomer concentration leads to insoluble CD polymers. Immobilized CD polymers are obtained by covalent cross-linking between CDs and some insoluble matrix [184]. Water-soluble CD polymers have more solubility than natural CDs and can solubilize the drug by both inclusion and noninclusion complex formation. CD polymers are long known to have a variety of industrial benefits [203-206], and various review articles provide detailed insights into the applicability of CD as a polymer [147, 198, 207, 208].

9.6.3 CDs and Nanotechnology

In the field of nanotechnology, CDs are used to create various supramolecular architectures, such as nanosponges, nanomicelles, nanovesicles, and nanomedicines [153]. Limitations of native CDs are mainly related to solubility and expensive and time-consuming separation. However, these limitations can be overcome with the use of CD polymers. CD-based nanosponges are widely used water-insoluble polymers with high porosity; therefore, they attain a sponge-like structure with a cavity to carry molecules [209]. Biodegradable and biocompatible polymers, such as polyglycolic acid, polyacrylic acid, polypeptides, and polysaccharides, are commonly used to make nanoparticles. Most of these polymeric materials are used to produce nanosponges; however, polysaccharides are most often used due to their nontoxic and hydrophilic nature and low cost. Polysaccharides can also be modified to improve their interaction with living tissues and are, therefore, often used as nanomedicines. Amphiphilic CD nanoparticles are also widely used and offer advantages, such as improvement in drug loading capacity and, most importantly, self-assembly without the requirement for surfactant [144]. Amphiphilic CD nanoparticles are formed by grafting an aliphatic carbon chain having an amide or ether group to the CD, with the carbon chain providing an extra area for interaction with drug molecules apart from the cavity. Amphiphilic CD nanoparticles are often used in tumor drug delivery and possess better interacting ability with biological membranes due to their amphiphilic nature [144].

9.6.4 Role of CDs in Biomedicine

The earlier role of CDs was just as an excipient. But with recent discoveries, scientists began to realize its importance in medical applications, such as API and biomedical technologies. A recent study described the potential role of CD derivatives as a carrier for siRNA delivery [210]. Few CD derivatives are approved for human use, and some, such as TRIMEB (heptakis-2,3,6-tris-O-methyl-*β*-CD) and SBE-*β*-CD (sulfobutylether *β*-CD), are under trial. The possible role of CD in medicine was first described in a study of sulfonated CDs that hindered HIV replication; however, the medicine was not approved due to the development of resistance by HIV against these sulfonated CDs [140, 211].

Another CD derivative with cholesterol sequestering ability, DIMEB (heptakis-2, 6-di-O-methyl-*β*-CD), was also developed; some others are still under toxicological studies. Of them, HP-*β*-CD (2-hydroxypropyl-*β*-cyclodextrin) is a promising candidate; however, more studies are required for its approval in HIV therapy. In vitro studies performed using influenza virus membrane demonstrated that RAMEB (randomly methylated *β*-CD) also has promising cholesterol sequestering ability [212]. Terpenic *β*-CDs inhibit infection by blocking haemagglutinin and preventing virus entry into the host cell. Currently, several studies are focusing on the development of mRNA vaccines against COVID-19. CDs can be used as an effective conjugate to encapsulate the mRNA because the naked mRNA is susceptible to degradation by RNases and the innate immune system. Encapsulation not only protects the mRNA but also assists in its entry into the cells by escaping the endosomes [213]. RAMEB was also reported as a promising candidate in various studies for controlling dengue [214], and later Braga [211] reported a role of RAMEB in treating leishmaniasis. Furthermore, CD sulfate with a 16.9 or greater degree of substitution was effective against malaria, but further studies are required for its final approval.

Some studies have reported that HP-*β*-CD is a good cholesterol sequester, thereby providing a ray of light to remove these substances from clogged arteries to treat atherosclerosis (a heart disease characterized by the accumulation of cholesterol that results in blocked arteries). NPD or Niemann–Pick disease (type-c) is an incurable brain disease [215] caused by a defective NPC-1 gene. The product of *NPC-1* plays a role in the transportation of water-insoluble compounds, such as sphingolipids and cholesterol. These insoluble compounds get accumulate in the brain leading to this disease. Studies on HP-*β*-CD in cats and mice have demonstrated significant results in the removal of these accumulated compounds from brain cells. Sugammadex, a *γ*-CD derivative (primary hydroxyl side of *γ*-CD is perfunctionalized with sulfanylpropanoic acid) induced by rocuronium bromide, vecuronium bromide, and pancuronium bromide, is an EMA and FDA approved drug for neuromuscular blockade reversal. This CD-drug complex is administered intravenously during surgery and is rapidly cleared from the patient's body post-surgery, unlike the conventional acetylcholinesterase inhibitors [211].

Biomedical technology studies have demonstrated that *β*-CDs have a role in implants of the biomimetic cornea [216]. CDs also help in the bioengineering of collagen in a controlled manner by enabling desired cross-linking. CDs mask certain amino acids during the fibrogenesis process, thereby preventing excessive cross-linking. BMPP-2 and TGF-*β* are the growth factors with a role in directing stem cells toward osteochondral cell formation; their release can be regulated in a controlled manner after the formation of an inclusion complex with *β*-CDs. Thus, CDs play a role in the treatment of arthritis and cartilage degeneration as well [217].

9.6.5 CDs and Textiles

In the textile sector, CDs find various applications, mainly in the field of bioactive textiles. A new field called cosmetotextiles has emerged that encompasses microencapsulations, cosmetics, and textile fields. Traditionally, various chemicals, such as phenolics, formaldehyde derivatives, and antibiotics, were used as antimicrobial agents in the production of textile fibers. However, most were not eco-friendly and, therefore, a new strategy utilizing natural products and green chemistry concepts, is required. CDs also provide an alternative in textile finishing because of their inclusion-complex-forming capability. In brief, CDs play a role in binding to some polymers and fibers to reduce their odor, in the controlled release of aromatic substances; moreover, they also anchor to substances with antimicrobial properties and act as a mosquito repellent [153]. Some of these applications are presented in Table 10.

Table 10 Applications of CDs in medical textiles.

Type of Textile	CD Formulation	Biological Role	Reference
Wool	Monochlorotriazine (MCT) *β*-CD (commercial MCT-*β*-CD product under trading name Cavasol W7) MCT is made available by Wacker-Chemie Company, Munchen, Germany) and is complexed with silver nanoparticles and triclosan	The antibacterial efficiency exceeds 75% after 15 washing cycles	Ibrahim et al. [218]

Cotton	CD loaded with silver (I) ions	Exhibited excellent antimicrobial property with no *E. coli* growth up to 7 days of observation	Bajpai et al. [219]
Pajamas made of knitted cotton	MCT *β*-CD complexed with menthol, *Viola tricolor Herba*, propolis, and hydrocortisone acetate	Used as a clothing comfort for skin sensitivity of patients with atopic or contact dermatitis	Radu et al. [220]
Cotton	Complex of *β*-CD with cypermethrin and prellethrin	Protection against mosquitoes	Abdel-Mohdy et al. [221]
Cotton	Complex of *β*-CD with permethrin and N, N-diethyl-m-toluamide	Protection against mosquitoes	Romi et al. [222]
Cotton	Complex of MCT *β*-CD with limonene	Protection against mosquitoes	Hebeish et al. [223]
Cotton	Complex of *β*-CD with permethrin and bioallethrin or grafting with glycidyl methacrylate	Protection against mosquitoes	Hebeish et al. [224]

9.6.6 CDs and Separation Process

Because CDs and their derivatives can differentiate isomers, enantiomers, and functional groups on a molecule very efficiently, they have been widely employed in the field of drug separation as a chiral selector or as an additive [181]; some of these applications are mentioned in Table 11. The ability to form inclusion complexes with specific molecules enables the usage of CD in this field. Because CD derivatives possess either ionic or nonionic functional groups, they are accordingly used in different chromatographic processes, such as gas chromatography, liquid chromatography, capillary chromatography, electrokinetic chromatography, and supercritical fluid chromatography. CDs are used as a ligand for sorption or as the chemically bounded ligand in the immobile or stationary phase in the separation process. They can also be added as buffer modifiers for the chiral separation of drugs and other chemicals. CDs are also used for the purification of CGTases [153].

Table 11 Role of CDs in the separation process.

Drug Name	**CD Used**	**Result**	**Reference**
Vinca alkaloids (Vincamine, vinpocetine, and vincadifformine)	Native CDs and their derivatives	Successful enantioseparation of three vinca alkaloids by using CDs and CD derivatives as chiral selectors.	Sohajda et al. [225]
Resibufogenin and cinobufagin	*γ*-CD	An effective separation method was developed wherein *γ*-CDs were added as mobile phase additives in a reverse phase HPLC.	Xing et al. [226]
Iodiconazole	HP-*γ*-CD	A capillary electrophoresis strategy	Li et al. [227]

		was developed for chiral separation of iodiconazole and structurally related triadimenol analogs.	
Racemic-trans-delta-δ-Viniferin (TVN)	HP-*β*-CD	An effective separation of TVN isomers by using a preparative chiral high-speed counter-current chromatography (HSCCC) method	Han et al. [228]

9.6.7 CDs in Food and Nutrition Industry

CDs are long known to be safe for oral consumption because they do not exhibit any toxic effects and are well tolerated by the human body [11, 229-231]. Therefore, their use as a flavor stabilizer, for removing undesirable compounds, and for getting rid of unpleasant tastes and odor has increased [11, 181, 232]. The encapsulation of molecules within CD is responsible for such characteristic features, leading to prolonged shelf life. For example, immobilized *β*-CD in glass beads assists in the removal of cholesterol from milk by 41% [233]. Some of these applications are listed in Table 12. CDs also improve barrier properties, such as the diffusion rate of volatile compounds, which in turn lead to improvements in food quality. AIT (allyl-isothiocyanate), an antifungal compound in a Japanese plant extract called wasabi, has various applications after encapsulation with CDs. This complex prevents AIT oxidation and lowers its volatility. Currently, the AIT-CD complex is used in various packaging materials, such as nylon films, tablets, and polyethylene [153].

Table 12 Application of CDs in the food sector.

Food Properties	**CD Used**	**Result**	**Reference**
Removal of smell from goat milk and yogurt	*α*-CDs, *β*-CDs, and *γ*-CDs	Removal of goat smell (effect of *β*-CD was highest) without hampering nutritional benefits.	Young et al. [234]
Removal of cholesterol (unwanted food substance)	*β*-CDs	Successful removal of cholesterol from food products.	dos Santos et al. [235]
Lowers the effects of fat-containing meals in individuals	*γ*-CDs	Alleviation of acute post-prandial response by reducing the triglyceride levels in the blood.	Jarosz et al. [236]
Mandarin juice with added goji berry juice and pomegranate extract (enhancement in desirable food properties)	*β*-CDs and HP-*β*-CDs	Increase in antioxidant activity, color intensity, vitamin C levels, and retinol equivalents was observed in juices containing HP-*β*-CDs.	Navarro et al. [237]
Extraction of polyphenols (plant-based nutrients)	Methyl-*β*-CDs	Combination of a novel deep eutectic solvent with methyl-*β*-CD resulted in efficient polyphenol extraction from Olea europaea leaves.	Athanasiadis et al. [238]

9.6.8 CDs in Environmental Science

CDs are used in the field of environmental science for various applications, such as the removal of pollutants from soil, air, and wastewater and the enhancement of organic contaminant solubility. The use of CDs in this field is desirable due to their low cost and biodegradability. CDs can form soluble and insoluble polymers, such as hydrogels, nanosponges, and various cross-linked substances. For example, Lukhele et al. [184] demonstrated the role of insoluble CD polymer in wastewater treatment. Singh et al. [239] utilized *β*-CDs to reduce the concentration of cyclic hydrocarbon pollutants, such as phenol, benzene, and p-chlorophenol, in the wastewater. Effective removal of gaseous effluents has been demonstrated by Szejtli [240]. Furthermore, the role of CD was also demonstrated in risk reduction technologies (to remove and destroy contaminants) through in-situ treatments. Gruiz et al. [241] reported the effectiveness of RAMEB in the removal of compounds, such as trichloroethylene, PCB, and PAH. Electrospun nanofibers are also widely used in the removal of pollutants. Celebioglu and Uyar [242] reported the removal of volatile organic compounds by using nanofibers as air filters. Moreover, Singh et al. [239] prepared a water-soluble encapsulation of azadirachtin A within the CD carrier, which was used as an insecticide formulation.

9.6.9 CDs in Catalytic Processes

Enzymes catalyze various biological reactions; however, real enzymes function only at the desired pH and are quite fragile to handle. Currently, biomimetic enzymes or artificial enzymes that mimic real enzymes are widely used [243-245]. A real enzyme performs two functions, i.e., binding to the substrate with a proper orientation and reaction catalysis. Therefore, to be used in the place of real enzymes, CDs should have these two essential properties. To achieve this, CD derivatives should be developed [246]. Because the CD structure consists of a hollow cavity, the addition of functional groups, such as flavin and aldehydes, will enable them to catalyze a reaction.

D’ Souza [243] developed an artificial enzyme for redox reactions by exclusively modifying the 2’, 3’ and 6’-OH group of a CD by the addition of the 4-methylamino-3-nitrobenzene group. In another study, Fenger and Bols [247] attached aldehydic groups to -OH of CDs, and the complex so formed exhibited Michaelis Menten kinetics similar to that of oxidase enzymes for catalyzing the oxidation of aminophenols. Glutathione peroxidase family (GPx) enzymes catalyze a reaction to remove hydrogen peroxides, but native GPx enzymes have disadvantages, such as poor availability, less stability, and antigenic property. Therefore, CDs can be a good alternative. In a study, substitutions at 2’ and 6’-OH positions with Te/Se groups led to appreciable results in reaction catalysis [248]. Therefore, studies on CD chemistry are required to explore the potential of CDs as a biomimetic enzyme.

However, apart from the role of CDs as a biocatalyst, they are also utilized in various transition metal-based catalytic reactions. Hapiot et al. [249] reported the usage of CDs in developing unconventional reaction media, such as thermoresponsive CD-based hydrogels and low-melting mixtures (LMMs). The use of these hydrogels in various transition metal catalytic reactions is advantageous over conventional catalytic systems because of characteristics, such as increased catalytic activity, catalyst stabilization, and catalyst recyclability [249]. Furthermore, CDs perform multiple roles during transition metal-based catalytic processes [250, 251]. Noël et al. [250] provided insights into various roles of CDs in catalytic processes involving water dispersed or

immobilized metal nanoparticles. In a study, CD-based metal nanoparticles (as catalysts) demonstrated high activity, stability, and recyclability because they can be used as a reducing agent of metal precursors and to form supramolecular hydrogels, which is a designated catalytic system that provides space for metal nanoparticles to get embedded [250]. Hapiot et al. [251] also discussed several roles of CDs, including catalyst activators and building blocks for encapsulation of catalyst, the advantage of CD-based catalysts (in aqueous media) in the ring-opening metathesis polymerization process, and the role of CD-dimer as a reaction platform (one CD cavity can include a substrate into it and the other can include an organometallic catalyst simultaneously) among others.

10. Conclusion

CGTase is a starch modifying enzyme belonging to the same glycosyl hydrolase superfamily as that of alpha and maltogenic amylases. It is an extracellular enzyme induced in the presence of starch (or other substrates) and is ubiquitously produced by all domains of life. CGTase is a multifunctional enzyme that catalyzes various reactions, such as cyclization, coupling, disproportionation, and hydrolysis. Cyclization is the major reaction catalyzed by CGTase, wherein the starch is converted to CDs. CGTase-catalyzed CD synthesis results in the mixture of various *α*-CDs, *β*-CDs, and *γ*-CDs, as well as large ringed CDs in some cases. As evident from the diversity of CGTase-producing organisms, the most well-characterized CGTases are *β*-CGTases, followed by some *α*-CGTases and *γ*-CGTases. CD production is an enzyme-mediated process, and only two commercial CGTases (Toruzyme and Amano CGTase) are available in the market. CGTase is applied in the commercial production of CDs and various transglycosylation products that find immense applications in various industrial sectors, such as food, pharmaceuticals, textiles, cosmetics, and agrochemical sectors. However, industrial production of CDs is limited by several challenges, such as the separation of a specific CD from mixtures. Moreover, product inhibition of CGTases and utilization of produced CDs in the coupling reaction also limit CD yields. More insights into the structure-function relationship of CGTases are required to overcome the issue of product inhibition. Moreover, most natural CGTases are thermolabile, and conversion reactions require high temperatures, thereby introducing the requirement for thermostable enzymes. These issues are being resolved either by protein engineering or by identifying novel CGTases with desired features from natural sources.

As evident from CD applications at the current time, the demand for CD and its application-based products will elevate in the future. The global CD market is distributed in food, cosmetics, pharmaceuticals, bakeries, beverages, and agriculture industries. As per some studies, the global CD market is estimated to rise from 180 US$ million in 2019 to 210 US$ million by 2024 and nearly $390 million by 2027 because of the large number of process and application patents available for CDs. Because CDs have a large market share, robust CGTase enzymes with high product specificity should be developed.

Acknowledgements

Kuldeep Saini would like to acknowledge the Council of Scientific and Industrial Research (CSIR) for providing a Senior Research Fellowship and Vinay Mohan Pathak duly acknowledges his VN Bakshi Postdoctoral fellowship.

Author Contributions

All the authors have read and contributed to this publication. Kuldeep Saini has written the initial draft, compiled all the data, and edited the complete manuscript till finalization. Vinay Mohan Pathak has written the enzyme production portion in the initial draft and has edited the final draft. Arpit Tyagi has written some portion of the cyclodextrin application in the initial draft. Prof. Rani Gupta has provided valuable ideas and suggestions for framing the initial draft and edited the drafts till manuscript finalization.

Competing Interests

The authors declare that they have no conflict of interest regarding this publication.

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