A review on immobilization strategy of cyclodextrin glucanotransferase producing *Escherichia coli* as whole cell biocatalyst

A A Abdullah¹,², N Z F N Azam², C W S R Mohamad² and R M Illias³

¹Medical Devices and Life Sciences Cluster, Sport Engineering Research Centre, Centre of Excellence (SERC), Universiti Malaysia Perlis (UniMAP), Perlis, Malaysia
²Faculty of Electronic Engineering Technology, Universiti Malaysia Perlis (UniMAP), Perlis, Malaysia
³Department of Bioprocess Engineering, School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, Skudai, Johor, Malaysia

E-mail: azamimi@unimap.edu.my

Abstract. This paper reviewed the immobilization strategy on cyclodextrin glucanotransferase (CGTase) producing *Escherichia coli* as whole cell biocatalyst. Biotransformation of insoluble substrate such as starch and lignocellulosic biomass with whole cell leads to the application of biological steps to chemocatalysis, which makes the whole process more effective. In this whole cell biocatalysis process, the excretion of the enzymes into the extracellular space by the cell is crucial. However, the use of free whole cell biocatalysts especially *Escherichia coli* often face with problem related to the cell lysis and stability. Due to that, the biotransformation process becomes inefficient. Thus, in this study the immobilization strategy was investigated to overcome the bottle necks mentioned. First, this study began with a focus in *Escherichia coli*, immobilization techniques, cyclodextrin, cyclodextrin glucanotransferase and recombinant *Escherichia coli*, in which can give a better understanding and a bigger picture in order to overcome the bottle necks mentioned. The different immobilization strategy and condition identified expected to improve the whole cell stability and reusability which lead to efficient biotransformation process. The output of the research also will elucidate the mechanism on how different immobilization material, strategy and cultural conditions improve the overall cell performance.

1. Introduction

This paper reviewed on the study regarding immobilization on cyclodextrin glucanotransferase (CGTase) producing *Escherichia coli* as whole cell biocatalyst. Starch is one of the components from massive amount of biomass readily available here in Malaysia, may it be fresh or waste type of biomass. Cyclodextrin glucanotransferase (CGTase) is one of the best-known enzymes that is able to react positively with starch. Starch is a biomass hence it is a very good practice to reduce biological waste. The most widely noted reaction of CGTase with starch is the production of cyclodextrin (CD) through intramolecular transglycosylation, which is one of the four reactions the enzyme CGTase is capable to catalyze. Cyclodextrin have many potential applications in wide range of industries including pharmaceuticals [1–9], food [10–14], cosmetic [15–20] and textile processing industries [21–26]. CGTase is usually produced by *Bacillus sp.* so, this will give an alternative since recombinant...
Escherichia coli is easily produced and obtained. This study will briefly discuss on cyclodextrin (CD), CGTase produced by Escherichia coli, immobilization techniques on Escherichia coli and the possible work of genome analysis of its chemotaxis, motility and also the adhesion properties by Escherichia coli in order to understand its ability. The purpose of this project is to propose a new technique based on genome analysis in producing CGTase from Escherichia coli.

2. Cyclodextrin
Cyclodextrins are cyclic glucose oligomers, which can transform water-soluble complexes of small molecules, but with large portions of compounds. Cyclodextrins are used for different purposes in pharmaceutical applications, including to improve the drugs bioavailability. One of the main interests in cyclodextrins is the use of polymers-cyclodextrin as a remarkable potential for nucleic acid delivery [27]. Cyclodextrins have many functional uses, and over the past few years they have gained more significance. This can be seen by the growing number of patents in particular. Since microencapsulation reflects inclusion, this phenomenon contributes in particular to the stabilisation of toxic substances that are responsive and enhances their handling and dosage. Other than pharmaceutical applications, there is also a remarkable agricultural application of cyclodextrins [28]. In another study, the researchers focused on the recent use of cyclodextrins in medicine particularly in oral drug delivery and delivery of protein, where the simultaneous use of different cyclodextrins will improve the main purpose of each host molecule in the delivery of oral drugs. In addition, cyclodextrins that are peracylated or regioselectively acylated may have wide applicability and can serve as novel hydrophobic or amphiphilic carriers of water-soluble drugs. The ionizable cyclodextrins or hydrophilic cyclodextrin will improve drug absorption in peptide and protein delivery, while hydrophobic cyclodextrins can be utilized in preparations of slow release [29]. As a carrier for colon targeting, the cyclodextrin biodegradation characteristic can be useful, and cyclodextrin may serve as a colon drug delivery [30]. In a recent study, synthesised free-diffusing photo-cross-linkable Ac-β-CDs (acyrloyl β-cyclodextrin, on average 1.5 acryloyl groups per Ac-β-CD molecule, i.e. a fraction of Ac-β-CDs carry more than one acryloyl group) are an essential element for the production of dynamic gelatin hydrogels, which can be used as drugs for the treatment of difficult bone effects [31]. This is only one of many examples of the specific use for medicinal purposes of cyclodextrins.

3. Cyclodextrin glucanotransferase (CGTase)
The formation of CD by intramolecular transglycosylation, which is one of the four reactions that the enzyme CGTase is capable of catalysing, is the most commonly noted reaction of CGTase with starch [32]. As described previously, Bacillus sp. normally produces CGTase. However, the use of recombinant Escherichia coli is an alternative means of generating CGTase. This study would therefore like to review a few methods conducted by other researchers who performed this alternative approach. A research was conducted by Su et al. to generate CGTase via the fermentation process using Escherichia coli as the host [33]. The investigation of CGTase development was performed from Escherichia coli containing the pET28b(+)-CGTase recombinant plasmid [34]. For protein expression, Escherichia coli BL21 (DE3) and pET28b(+)CGTase were used as host and plasmid [34]. Another research was carried out to see the possible pathways for increased alpha-CGTase secretion from the periplasmic compartment into the culture medium [35]. In this review, it is understood that it was found that these chemicals, SDS, Na+, glycine and Ca2 +, could boost the extracellular development of the enzyme in order to produce high quantity of recombinant alpha-CGTase from P. macerans [35].

A study by Cheng et al. suggested a new method of processing Escherichia coli CGTase [36]. This research used the fed-batch fermentation strategy for the development of high cell density Escherichia coli cultivation and extracellular production of Paenibacillus macerans JBF05-01 recombinant R-CGTase [36]. For all fermentation experiments, the recombinant E. coli BL21-(DE3) carrying the alpha-cgt gene of P. macerans JBF05-01 (constructed in their laboratory) was used. The plasmid was extracted from the vector pET-20b(+) with OmpA signal peptide and the analysis results showed that a two-stage
feeding strategy with complex sources of nitrogen (peptone and yeast extract) was sufficient for the development of high cell density cultures and proteins [36].

Another research by Liu et al. suggested other method or technique for the development of Escherichia coli CGTase, such as systematic optimization of codon use [37]. This study used, as in the previous study, the alpha-cgt gene from Paenibacillus macerans JFB05-01 to E. coli BL21-(DE3). But in this analysis, with systematic codon optimization, the mature alpha-cgt gene from Paenibacillus macerans JFB05-01 was redesigned to preferentially fit codon frequencies of Escherichia coli without modifying the amino acids sequence [37]. By some magnitude, the synthetic gene (codon optimised alpha-cgt gene) provided a much greater protein yield than the wild type [37].

Co-expression of folding accessory proteins, molecular chaperones, and human peptidyl-prolyl cis-trans isomerase (PPIase) is another technique or process for generating CGTase from Escherichia coli [38]. This strategy shows increased development of active Bacillus macerans cyclodextrin glycosyltransferase (CGTase), otherwise expressed primarily as an inclusion body in recombinant Escherichia coli. Human PPIase followed by co-expression of DnaK-DnaJ-GrpE together with GroEL-GroES was found to be the best partner for soluble expression of CGTase. The reason why human PPIase found to be best partner for CGTase enhancement is due to dual functions of chaperone and peptidyl-prolyl cis–trans isomerization. Human PPIase co-expression appeared to produce such a substantial change because of the dual roles of chaperone and peptidyl-prolyl cis-trans isomerization [39].

By reviewing other studies, producing CGTase from Escherichia coli is a very promising area and many of those studies used E. Coli BL21(DE3) as host.

4. Recombinant Escherichia coli
In order for a host to produce a desired recombinant protein (in this case CGTase enzyme), the host must be studied and carefully picked and engineered first to ensure the production to be optimized and the desired outcome come as a result.

There are well known benefits of using Escherichia coli as the host organism. First, it has unmatched kinetics of quick growth. Second, cultures with high cell density are readily achieved. Third, it is possible to create rich, complex media from readily available and inexpensive components. Forth, fast and simple transformation with exogenous DNA [40].

In order to use Escherichia coli as the host for the desired protein development, certain factors must be taken into account: DNA sequences involved in transcription, translation, and DNA sequences involved in protein translocation to the periplasm [41]. Translocating the recombinant proteins into the periplasm is wise and the reasons are: (1) the oxidising environment promotes disulfide bond formation, (2) it includes just 4% of the total cell protein (~100 different proteins), (3) less protein degradation occurs, and (4) fast osmotic shock purification [41].

There are several factors that could cause the low expression of the recombinant gene: (1) mRNA stability, (2) occurrence of secondary structures near the 5'end of the mRNA, (3) unusual codons and (4) poor sequence of Shine Dalgarno [41]. In the case of no or low output of recombinant protein, we must consider: (1) protein toxicity, (2) codon bias, and (3) batch cultivation limiting factors [41]. There are a few variables that can come into play in the formation of inclusion bodies to trigger it and ways of troubleshooting it, and they are: (1) disulfide bond formation, (2) co-expression or chemical chaperones of chaperones and cofactor supplementation of chaperones, (3) slowing down the rate of production [41].

We can see from previous research that the E. Coli BL21(DE3) strain was used primarily to generate CGTase. This is because the target protein of expression of gene is powered by the T7 RNA polymerase (P) bacteriophage, which transcribes much faster and more efficient than E. coli RNAP [42].

The development of the model single-chain variable antibody fragment (scFv) BL1 in the periplasm of E. coli can also be optimised. It can be done by modulating the level of gene expression using the Lemo setup. The DsbA signal sequence was used to target scFv BL1 to the Sec-translocon in the
cytoplasmic membrane via the SRP-targeting pathway. This approach results in the creation not only of more biomass, but also of substantially more secreted target protein per biomass unit [42].

5. Immobilization Techniques

Immobilization is a method of immobilising the enzymes by adding them to an inert, insoluble substrate, such as calcium alginate. It is produced by reacting to a mixture of enzyme of sodium alginate and calcium chloride solution. In conditions such as pH or temperature, this can provide improved resistance to changes. For this particular project, this approach was chosen because Escherichia coli appears to undergo cell lysis and immobilisation is a method to avoid that.

Four approaches, namely (1) non-covalent adsorption and deposition, (2) physical trapping, (3) covalent binding, and (4) bio-conjugation, are traditionally used for enzyme immobilisation. There are few methods that can be done by researchers to do the immobilization technique and it depends on the researchers themselves on how to do it. In this subtopic, we would like to discuss few previous studies that were related to immobilization and Escherichia coli specifically.

A new method for the development of rationally engineered biocatalytic surfaces based on BIND technique was proposed in a study. This method exploited the E. coli curli framework for the establishment of a functional nanofiber network capable of enzyme covalent immobilisation [43]. There are other techniques to immobilize Escherichia coli. One research indicated the use of the antimicrobial peptide cecropin P1 in which Escherichia coli K12 and O157: H7 cells were stuck on the well surfaces of the microtiter plate [44]. But only those strains of Escherichia coli can be immobilised by this technique. The researchers performed immobilisation of whole Escherichia coli JY001 cells using barium alginate in another study, to generate itaconic acid using Escherichia coli JY001 [45]. The development of β-galactosidase from Escherichia coli K12 in another study indicates a new novel of immobilisation technique. It was achieved by immobilising artificial liposomes with β-gal- cyt b5 and could solve the issue of lysis [45]. Another analysis of the development of Escherichia coli inulase II using the immobilisation method of adsorption of inulase II on anion exchangers [46]. This was achieved by testing the resin charged with various anions (OH\(^{-}\), Cl\(^{-}\) and PO\(_4\)^{3-}\) and also researching the effect of pH equilibration [47]. In this immobilisation initiative, this approach may also be considered.

Last but not least, a study for producing penicillin amidase from Escherichia coli was done using immobilization technique. In this study the researchers used two methods of immobilization that are two crosslinking methods: (1) crosslinking with cyanuric chloride, and (2) crosslinking with glutaraldehyde and the granulation method [48]. Therefore, the granulation method steps in and the results showed less protein leaching and greater mechanical strength while the treatment of cyanuric chloride shows low performance in the whole cell immobilisation in comparison of the crosslinking method for glutaraldehyde [48]. The whole-cell immobilisation system shows a disadvantage where the proteins and other metabolites leached.

6. Genome analysis on motility, adhesion and chemotaxis in Escherichia coli

Motility, adhesion and chemotaxis plays a vital role of an organism in immobilization. This part of the study will focus on the genome analysis of an Escherichia coli on those properties mentioned in order to understand and maybe to enhance its performance in immobilization technique. This part will study on other research papers on motility, adhesion and chemotaxis of an Escherichia coli.

In response to stress, the chemotaxis and motility for machinery is generated, but the cells respond behaviourally only to decreasingly stressful changes in their micro-environments [49].

Flagellar plays a crucial function in mobility and motility in Escherichia coli. It is the primary mechanism for it to ‘swim’ through its ideal setting [50,51]. Many environmental factors have established the flhD operon at the apex of the flagellar regulon as the primary target of regulation [52]. The two genes consisting of the operon, flhD and flhC, are the products that form a heterotetrameric regulatory transcriptional complex, FlhD or FlhC [53]. In a study, researchers attempted to spontaneously insert IS5 and IS1 elements into the flhD operon’s regulatory region and increase expression and concluded that these factors could play a vital role in the motility of the organism as well as many other factors and
variables that could be taken into account and could also contribute to the diversity of the inter-and intra-population [54]. In *Escherichia coli* flagellar rotation, high levels of the second messenger cyclic dimeric GMP, c-di-GMP, cause a counter clockwise bias that results in smooth swimming [55]. It may well be understood that this is also a big part of the motility of *Escherichia coli*.

In other studies, it is known that potential factors associated with virulence, such as genes encoding flagellum expression and assembly, motility and chemotaxis, have been triggered by quorum sensing [53].

In *Escherichia coli*, there is another regulator (other than FlhDC regulon) that controls the flagellar regulon that is flagellum-specific sigma factor, $\sigma^F$ [54]. In this study, it also notes that motility could be regulated by carbon source in which the results showed, unanticipatedly, a gradual increase in motility activity with decreased carbon quality because there is a behaviour, which is called risk-prone foraging where the bacteria compromise and actively search for better conditions using the flagellar system [54].

Motility, chemotaxis and adhesion plays vital role between one another in immobilization of said bacteria. In this part, we will discuss previous research on the correlation between those properties in immobilization. Cyclic or c-di-GMP has been discovered as a main role in the decision between motile and sedentary forms of bacterial life [56]. YcgR is dedicated to c-di-GMP-dependent inhibition of motility through its interaction with flagellar motor proteins [57]. In a review, it is suggested that smooth swimming induced by c-di-GMP promotes the trapping of motile bacteria in semi-solid media and the attachment of liquid-grown bacteria to solid surfaces, while c-di-GMP-dependent mechanisms not involving YcgR further facilitate surface attachment where YcgR is a regulator of c-di-GMP that binds to the flagellum switch complex's FliG subunit [57]. The second c-di-GMP messenger is a critical component of the transformation from single-cell motile to surface-attached multicellular bacteria. Inhibition of cell motility and surface attachment requires this change [57].

In other study, the researchers tried to find the correlation between *Escherichia coli* motility and adhesion. It is found that during the transformation phase, *Escherichia coli* changes from motile-planktonic to the adhesive-sedentary “lifestyle” [52]. This transition is suggested to be regulated at two separate hierarchical levels by mutual inhibition of FlhDC or motility and $\sigma$ or adhesion regulation cascades, where motility gene expression and general stress response are inversely coordinated at the top level by $\sigma^W / \sigma^{FliA} / \sigma$ competition for core RNA polymerase and the FlhDC-controlled FliZ protein acting as an inhibitor of $\sigma^W$ and at $\sigma$. In *Escherichia coli*, this illustrates the inverse regulatory synchronisation of motility and curli-mediated adhesion.

In other studies, it has been shown that motility, not chemotaxis, is important for the development of biofilms where motility plays a role in both initial surface interaction and surface movement [52]. It is also stated that for initial surface attachment, type I pili (harbouring the man-nose-specific adhesin, FimH) is necessary and that mannose inhibits normal attachment [52]. Flagella plays a direct role in the formation of a biofilm for attachment to the abiotic surface, so motility may be sufficient to allow a bacterium to reach the surface. Last but not least, it is claimed that the initial attachment to abiotic surfaces of type I pili is critical.

7. Conclusion
Taking these studies into consideration, we could understand more about the roles played of motility, chemotaxis and adhesion properties of an *Escherichia coli* in performing the immobilization technique. From the comprehensive review, it is found that two genes; flhD and flhC, plays a vital role in *Escherichia coli* motility since they are the main mechanism for the flagellar to ‘swim’ to desired environment. These properties play a vital role in immobilization technique. Our future work will include the flhD and flhC genome analysis for the strategy of *Escherichia coli* immobilization in order to produce cyclodextrin glucanotransferase (CGTase). It is expected that the mutation of flhD and flhC can give positive effects on motility of *Escherichia coli*. By enhancing the formation of the flagella, the *Escherichia coli* may be able to attach easier to the support material used for the immobilization. This can be performed by identifying the pathway for the formation of these flagella and manipulate the pathway to increase the motility of *Escherichia coli*.
8. References

[1] Perinelli D R, Palmieri G F, Cespi M and Bonacucina G 2020 *Molecules* **3** 12 1023-35
[2] Loftsson T and Duchêne D 2007 *Int. J. Pharm.* **329**(1-2) 1-11
[3] Crini G, Fourmentin S, Fenyvesi É, Torri G, Fourmentin M and Morin-Crini N 2018 *Environ. Chem. Lett.* **16**(4) 1361-75
[4] Menezes P dos P, Andrade T de A, Frank L A, de Souza E P B S S, Trindade G das G G, Trindade I A S, Serafini M R, Guterres S S and Araujo A A de S 2019 *Int. J. Pharm.* **559** 312-28
[5] Jansook P, Ogawa N and Loftsson T 2018 *Int. J. Pharm.* **535**(1-2) 272-84
[6] Sikder M T, Rahman M M, Jakariya M, Hosokawa T, Kurasaki M and Saito T 2019 *Chem. Eng. J.* **355** 920-41
[7] Braga S S 2019 *Biomolecules* **9**(12) 801
[8] Conceição J, Adeoye O, Cabral-Marques H M and Lobo J M S 2017 *Curr. Pharm. Des.* **24**(13) 1405-33
[9] dos Santos C, Buera P and Mazzobre F 2017 *Curr. Opin. Food Sci.* **16** 106-13
[10] Ho S, Thoo Y Y, Young D J and Siow L F 2019 *Food Chem.* **275** 594-99
[11] Nedovic V, Kalusevic A, Manojlovic V, Levic S and Bugarski B 2011 *Procedia Food Sci.*
[12] Simionato I, Domingues F C, Ferreira M R, Guterres S S, Trindade I A S, Serafini M R, Guterres S S and Araujo A A de S 2019 *Int. J. Pharm.* **559** 312-28
[13] Ho S, Thoo Y Y, Young D J and Siow L F 2017 *LWT - Food Sci. Technol.* **85** 232-39
[14] Martínez-Delgado A A, Khandual S and Villanueva–Rodriguez S J 2017 *Food Chem.* **225** 23-30
[15] Sakulwech S, Lourith N, Khandual S and Villanueva–Rodriguez S J 2017 *Food Chem.* **225** 23-30
[16] Mori T, Tsuchiya R, Doi M, Nagatani N and Tanaka T 2019 *J. Incl. Phenom. Macrocycl. Chem.*
[17] Lee J and Park S 2019 *Asian J. Beauty Cosmetol.* **9**(3) 105-112
[18] Allahyari S, Trotta F, Valizadeh H, Jelvehgari M and Zakeri M 2019 *Expert Opin. Drug Deliv.* **16**(5) 467-79
[19] Zhang B, Huang J, Liu K, Zhou Z, Jiang L, Shen Y and Zhao D 2019 *Ind. Eng. Chem. Res.* **58**(43) 19767-71
[20] Bochot A and Piel G 2011 *Cyclodextrins in Pharmaceutics, Cosmetics, and Biomedicine: Current and Future Industrial Applications*
[21] Lis M J, Carmona Ó G, Carmona C G and Bezerra F M 2018 *Polymers (Basel)* **10**(12) 1324
[22] Singh N and Sahu O 2018 *The Impact and Prospects of Green Chemistry for Textile Industry* 83-105
[23] Rehan M, Mahmoud S A, Masih M H M and Youssef B M 2020 *React. Funct. Polym.* 104573
[24] Bhaskara-Amrit U R, Agrawal P B and Warnekeskerken M M C G 2011 *Autex Res. J.*
[25] Azizi N, Ben Abdelkader M, Chevalier Y and Majdoub M 2019 *Fibers Polym.* **20**(4) 683-89
[26] Mehraz L and Nouri M 2020 *J. Nat. Fibers*
[27] Uekama K and Otagiri M 1987 *Crit. Rev. Ther. Drug Carrier Syst.* **3** 1 1-40
[28] Zavareze E da R, Kringle D H and Dias A R G 2019 *Advances in Food and Nutrition Research* **88** 85-128
[29] Irie T and Uekama K 1999 *Adv. Drug Deliv. Rev.* **36**(1) 101-23
[30] Hirayama F and Uekama K 1999 *Adv. Drug Deliv. Rev.* **36**(1) 125-41
[31] Feng Q, Xu J, Zhang K, Yao H, Zheng N, Zheng L, Wang J, Wei K, Xiao X, Qin L and Bian L 2019 *ACS Cent. Sci.* **5**(3) 440-50
[32] Benavent-Gil Y, Rosell C M and Gilbert E P 2021 *Food Hydrocoll.* **112** 106316
[33] Su L, Jiang Q, Yu L and Wu J 2017 *C Microbiol. Cell Fact.* **16**(1) 1-11
[34] Yang Y N, Shan W X and Wang P W 2017 *J Biotechnol* **7** 3 1-10
[35] Ding R, Li Z, Chen S, Wu D, Wu J and Chen J 2010 *Process Biochem.* **45** 6 880–86
[36] Cheng J, Wu D, Chen S, Chen J and Wu J 2011 *J. Agric. Food Chem.* **59** 8 3797–802
[37] Liu H, Li J, Du G, Zhou J and Chen J 2012 *J. Ind. Microbiol. Biotechnol.* **39** 12 1841–49
[38] Kim S G, Kweon D H, Lee D H, Park Y C and Seo J H 2005 *Protein Expr. Purif.* **41** 2 426–32
Acknowledgements

This work was funded by Universiti Malaysia Perlis (UniMAP) Collaborative Research Grant (CRG) 9023-00006.