

# Enhancement of $\beta$ -cyclodextrin Production and Fabrication of Edible Antimicrobial Films Incorporated with Clove Essential Oil/ $\beta$ -cyclodextrin Inclusion Complex

Mohamed G. Farahat\*

Botany and Microbiology Department, Faculty of Science, Cairo University, 12613 Giza, Egypt

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Edible films containing antimicrobial agents can be used as safe alternatives to preserve food products. Essential oils are well-recognized antimicrobials. However, their low water solubility, volatility and high sensitivity to oxygen and light limit their application in food preservation. These limitations could be overcome by embedding these essential oils in complexed product matrices exploiting the encapsulation efficiency of  $\beta$ -cyclodextrin. This study focused on the maximization of  $\beta$ -cyclodextrin production using cyclodextrin glucanotransferase (CGTase) and the evaluation of its encapsulation efficacy to fabricate edible antimicrobial films. Response surface methodology (RSM) was used to optimize CGTase production by *Brevibacillus brevis* AMI-2 isolated from mangrove sediments. This enzyme was partially purified using a starch adsorption method and entrapped in calcium alginate. Cyclodextrin produced by the immobilized enzyme was then confirmed using high performance thin layer chromatography, and its encapsulation efficiency was investigated. The clove oil/ $\beta$ -cyclodextrin inclusion complexes were prepared using the co-precipitation method, and incorporated into chitosan films, and subjected to antimicrobial testing. Results revealed that  $\beta$ -cyclodextrin was produced as a major product of the enzymatic reaction. In addition, the incorporation of clove oil/ $\beta$ -cyclodextrin inclusion complexes significantly increased the antimicrobial activity of chitosan films against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella Typhimurium*, *Escherichia coli*, and *Candida albicans*. In conclusion, *B. brevis* AMI-2 is a promising source for CGTase to synthesize  $\beta$ -cyclodextrin with considerable encapsulation efficiency. Further, the obtained results suggest that chitosan films containing clove oils encapsulated in  $\beta$ -cyclodextrin could serve as edible antimicrobial food-packaging materials to combat microbial contamination.

**Keywords:**  $\beta$ -cyclodextrin glucanotransferase, *Brevibacillus*, RSM, optimization, immobilization

## Introduction

Microbial contamination of food products accelerates the deterioration process and increases the risk of food-borne illness caused by potentially life-threatening pathogens. Ordinarily, food contamination originates

superficially therefore, proper surface treatment and packaging is crucial for assuring food quality and safety [1]. Recently, edible films have gained much interest in preserving various food products, retaining their firmness and restricting weight loss that occurs due to loss of moisture [2–4]. Chitosan is an abundantly available biodegradable polysaccharide that demonstrates remarkable film-forming properties [5–7]. Furthermore, the edible films could be used as a vehicle for additives such as antioxidants and antimicrobials,

### \*Corresponding author

Tel: +20-1116080701, Fax: +20-235727556

E-mail: farahat@cu.edu.eg

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and allows increasing the shelf life of foodstuffs. Although synthetic chemical compounds have long been used as preservatives to inhibit a wide variety of food-borne pathogens, their potential negative impact on human health has prompted research on the use of naturally occurring alternatives. Thus, it has been an increasing concern in using natural antimicrobials including, bacteriocins from microbial sources [8, 9], animal-based proteins such as lactoferrin [10], and essential oils (EOs) derived from plants [11, 12]. Of these natural antimicrobial agents, plant-derived EOs have gained extensive popularity due to the growing social awareness and demand for safer products that present a more close-to-natural image. Being secondary metabolites produced by aromatic plants, EOs have shown potent antimicrobial effect against several pathogenic and spoilage microorganisms [13–16]. In particular, clove essential oil (CEO) has been reported as a potent antimicrobial agent against various microbes owing to the presence of eugenol as the major active constituent [17–19]. It has been suggested that eugenol exerts the bactericidal action by the destruction of the cytoplasmic membranes and altering the integrity of the cell membrane. The disruptive action of eugenol on cytoplasmic membrane increases the permeability and causes the losses of vital intracellular materials leading to bacterial death [20, 21]. Recently, the antimicrobial effect of CEO is thought to be due to the destruction of cell structure and inhibition of DNA and proteins synthesis [22]. Regarding antifungal activity, CEO and eugenol are found to reduce the quantity of ergosterol, a specific fungal cell membrane component, and inhibit the formation of germ tubes in *Candida albicans* [23]. Nonetheless, despite these auspicious properties, EOs have poor water solubility, intense aroma, high volatility and high sensitivity in the presence of light, and oxygen, which mainly limit their application as natural preservatives [24, 25]. Hence, encapsulation of EOs in cyclodextrins seems to be a promising, and efficient approach for overcoming these problems by masking the flavor of essential oils, protecting them against oxidation damage, and allowing for EOs to persist for longer time periods under a wide variety of environmental conditions [26, 27]. Cyclodextrins (CDs) are cyclic, water-soluble ring-shaped oligosaccharides, which are composed of several D-glucose units linked by  $\alpha$ -D-(1-4) linkages and

their three-dimensional structure considered as a truncated cone. The most common molecules are  $\alpha$ -CD,  $\beta$ -CD and  $\gamma$ -CD, which consist of six, seven and eight D-glucose units, respectively, that possess a cage-like supramolecular structure [28]. Because of their hydrophobic cavity and hydrophilic external surface, CDs exhibit the ability to interact with hydrophobic bioactive compounds and molecules (guest molecules), encapsulating them in their cavity, forming host-guest inclusion complexes (ICs) [28]. Besides their applications in encapsulation of EOs, CDs have numerous applications in the pharmaceutical, cosmetics, and textile industries. CDs are produced from the enzymatic conversion, degradation, and cyclization of starch by cyclodextrin glucanotransferase (CGTase) as a result of an intramolecular transglycosylation reaction [29–31]. The present investigation addresses the enhanced enzymatic production of  $\beta$ -cyclodextrin using cyclodextrin glucanotransferase derived from *Brevibacillus brevis* AMI-2 with emphasis on its application.

## Materials and Methods

### Sample collection

Mangrove sediments were collected from Abu Minqar Island, at the Red Sea (27°12'42.0"N 33°52'10.0"E). The top 5–10 cm of sediments were sampled using sterile disposable wooden spatula during low tides. The collected mangrove sediments were placed in sterile polyethylene bags and transported to the laboratory on ice.

### Isolation of CGTase-producing bacteria

CGTase-producing bacteria were isolated on Horikoshi II agar (1% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1%  $K_2HPO_4$ , 0.02%  $MgSO_4 \cdot 7H_2O$ , 1%  $Na_2CO_3$ , 0.03% phenolphthalein (PHP), 0.01% methyl orange and 1.5% agar) according to the method described previously [32]. Samples were suspended in sterile saline and vigorously vortexed for 15 min, and then serially diluted in sterile saline, and 100  $\mu$ l of each dilution were plated in a triplicate onto Horikoshi II agar plates. After 24–72 h of incubation at 37°C, yellowish colored zones were detected around the CGTase-producing colonies. These bacterial isolates were picked and re-streaked on the same medium. Discrete colonies were transferred to slants of the same growth culture medium without PHP

and methyl orange and maintained at 4°C.

**Inoculum preparation and culture conditions**

Modified Horikoshi II broth (20 g/l soluble starch, 5 g/l peptone, 5 g/l yeast extract, 1 g/l K<sub>2</sub>HPO<sub>4</sub>, 0.2 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, and 10 g/l Na<sub>2</sub>CO<sub>3</sub>) was used as a seed and production medium. Bacterial isolates were cultivated overnight at 37°C with shaking at 200 rpm. Cells were then collected by centrifugation at 6000 ×g for 5 min, re-suspended in a sterile phosphate buffered saline to give an optical density (OD<sub>600</sub>) reading of 1.2, and were used as inocula. The production medium was inoculated with a bacterial isolate (2% v/v) and cultivated in shake flasks (200 rpm) at 37°C for 72 h. Bacterial cells were removed by centrifugation at 21,000 ×g for 30 min at 4°C and the cell-free supernatant was used as a crude enzyme.

**Enzyme assay**

The CGTase activity was measured as β-cyclodextrin (β-CD) forming activity by the PHP method [33]. Fifty-microliters crude enzyme were added to 1 ml of 2% soluble starch in 50 mM Tris-HCl buffer (pH 7.0) and incubated at 55°C for 15 min. Afterwards, 4 ml of 4 mM PHP in ethanol and 125 mM Na<sub>2</sub>CO<sub>3</sub> (pH 11.0) were added and the color intensity was measured at 550 nm using a double beam UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). One unit of the CGTase activity was defined as the amount of enzyme that catalyzes the production of 1 μmol of β-CD per minute under the reaction conditions. A standard curve was prepared using β-CD in 50 mM Tris HCl buffer (pH 7.0) and the protein content was assayed by Bradford method [34]. The specific activity of CGTase was expressed as U/mg protein.

**Identification of bacterial isolates**

CGTase-producing bacteria were identified by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS). A fresh colony of each bacterial isolate was spotted into the target steel plate using a sterile toothpick. To each bacterial spot, 1 μl of 70% formic acid and 1 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid were applied and allowed to dry. Spectra were obtained using the Flex-Control MicroFlex LT mass spectrometer analyzed

using FlexControl 3.0 software (Bruker Daltonics GmbH, Germany) as previously described [35].

**16S rRNA gene amplification and phylogenetic analysis**

Genomic DNA was extracted and purified using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to manufacturer's protocol and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'CGGTTACCTTGTTACGACTT-3'). PCR was conducted in 50 μl reaction mixtures containing 25 μl OneTaq™ 2X master mix (NEB, England), 50 ng genomic DNA template, 0.4 μM of each primer, and nuclease-free water was added to make volume up to 50 μl. The amplification was processed with the following parameters: initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 68°C for 1.5 min for 32 cycles, and a final extension at 68°C for 5 min. Amplicons were purified using DNA clean & concentrator kit (Zymo Research) and sequenced at Macrogen (Korea). The forward and reverse DNA sequence reads were assembled using codoncode aligner software v12.1.2 and analyzed using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the EzTaxon-e server database [36]. Multiple sequence alignments were implemented utilizing ClustalW, and the phylogenetic tree was constructed using the neighbor-joining method using MEGA7 with bootstrap values based on 1000 replications [37].

**Optimization of CGTase production using response surface methodology (RSM)**

The central composite design (CCD) was used to analyze the interaction among six cultural factors and deter-

**Table 1. Range of variables of the central composite design.**

Factor	Unit	Coded levels	
		-1	+1
Starch concentration	%	1.0	5.0
Yeast extract concentration	g/l	0.1	5.0
Salinity (NaCl concentration)	%	0.5	3.0
Incubation period	h	48.0	96.0
Temperature	°C	30.0	60.0
pH		6.0	10.0

mine their optimal values (Table 1). The modeling was conducted using Design Expert (version 6.0.8, Stat-Ease, Inc., USA) statistical software including analysis of variance (ANOVA) that generated 90 experiments to obtain the interactions between the process variables and the response. To validate the statistical model, an experiment under the optimal condition predicted by the model was conducted and the actual experimental value was compared with the predicted one.

#### Purification and immobilization of CGTase

CGTase was purified by starch adsorption method with some modifications [38]. The most active strain designated AMI-2 was cultivated under the optimized conditions for enzyme production then cells were harvested by centrifugation at  $9400 \times g$  for 20 min at  $4^\circ\text{C}$ . Insoluble corn starch (5%) was added to the supernatant then ammonium sulfate was added to 20% saturation with continuous stirring at  $4^\circ\text{C}$  to allow enzyme adsorption for 2 h. The starch-adsorbed enzyme was collected by centrifugation at  $6000 \times g$  for 20 min at  $4^\circ\text{C}$  and the pellet was washed three times with phosphate buffer (50 mM, pH 7.5). Afterwards, the enzyme was eluted by adding 1 mM  $\beta$ -CD (in 50 mM phosphate buffer) with gentle stirring for 30 min at  $37^\circ\text{C}$ . After centrifugation at  $21,000 \times g$  for 30 min at  $4^\circ\text{C}$ , the supernatant was dialyzed against 10 mM phosphate buffer (pH 7.5) and freeze-dried. The lyophilized enzyme was immobilized into calcium alginate microspheres using endogenous emulsification method [39]. The operational stability of the immobilized enzyme was determined by quantifying its activity in consecutive cycles of repeated use of the enzyme.

#### Cyclodextrin production and product specificity of CGTase

To determine the type of CD produced by the immobilized CGTase, 100 ml of 10% soluble starch were added to the immobilized enzyme and incubated at  $50^\circ\text{C}$  for 4 h. Then the products were analyzed against standard solutions of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD by high-performance thin layer chromatography (HPTLC) using Camag<sup>®</sup> HPTLC system (Muttens, Switzerland).

#### Purification and crystallization of $\beta$ -cyclodextrin

$\beta$ -CD was purified from other impurities and crystal-

lized according to the method reported by Sophianopoulos and Warner [40]. Briefly, isopropanol was added to the produced CDs mixture to final concentration of 35% (v/v) in a stoppered conical flask and the pH of the mixture was adjusted to 11 by addition of NaOH (0.1 M). Afterwards, the solution was incubated in a water bath at  $70^\circ\text{C}$  for 20 h. After cooling, the mixture was neutralized to pH 7.5 with 0.1 M HCl, allowed to stand at room temperature for 48 h, and then the insoluble material was removed by filtration. Subsequently, isopropanol was added to the filtrate to final concentration of 60% (v/v) and the pH was raised to 11. After 20 h incubation at  $70^\circ\text{C}$ , the hot solution was filtered and neutralized to pH 7.5 and allowed to crystallize at  $4^\circ\text{C}$  for 5 days. Then  $\beta$ -CD crystals were collected by filtration and re-dissolved in isopropanol (35%, v/v) and the abovementioned processes were repeated 3 times. The final precipitated  $\beta$ -CD crystals were filtered and dried at  $70$ – $75^\circ\text{C}$  under vacuum. The purified  $\beta$ -CD was freeze-dried and used for the preparation of inclusion complex (IC) with clove essential oil (CEO).

#### Preparation of Clove oil/ $\beta$ -CD Inclusion Complex (IC)

The essential oil was extracted from *Syzygium aromaticum* flowers (clove) by steam distillation method [41]. The inclusion complex of CEO and  $\beta$ -CD was prepared using a co-precipitation method with minor modifications [42]. Briefly, 5 g of the purified  $\beta$ -CD was dissolved in 100 ml of an ethanol/distilled water solution (1:2, v/v) at  $60^\circ\text{C}$  then allowed to cool to  $40^\circ\text{C}$ . Five-hundred milligrams of CEO were dissolved in ethanol and added to the  $\beta$ -CD solution dropwise. After sonication at 90 W for 4 h, the solution was incubated overnight at  $4^\circ\text{C}$ . The precipitated CEO/ $\beta$ -CD complex was harvested by vacuum filtration and washed three times with 30% ethanol solution and dried in a vacuum oven at  $40^\circ\text{C}$  for 6 h. The dried complex powder was stored in an airtight glass desiccator at room temperature until use.

#### CEO/ $\beta$ -CD-Chitosan film formulation

The edible chitosan-based film incorporated with CEO/ $\beta$ -CD inclusion complex was prepared by dissolving 1 g of chitosan (CS) in 100 ml of acetic acid solution (1% v/v) with continuous stirring at  $30^\circ\text{C}$ . Then glycerol was added as a plasticizer at 30% (w/w of CS) and various

concentrations of the prepared CEO/β-CD complex were added to reach a final concentration 0.25, 0.5, 0.75, 1.0, 1.25 or 1.5% (w/v) [27]. Fifteen-milliliter volumes of the film solutions were poured in Petri dishes and dried at 37°C for 24 h, films without CEO/β-CD were used as control. The dried films were separated from the dishes and used for antimicrobial assays.

### Antimicrobial activity

Antimicrobial potential of CEO/β-CD-Chitosan film was tested against *Escherichia coli* ATCC 8739, *Salmonella enterica* subsp. *enterica* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* subsp. *aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus subtilis* (ATCC 6633), and *Candida albicans* (ATCC 10231) according to method reported previously [43]. Regarding *B. subtilis*, spores suspension was prepared and harvested following the method described by previously [44]. For antibacterial testing, the edible β-CD/EO-Chitosan film with 2 cm diameter was placed into a sterile Petri dish. The surface of the film was inoculated with 100 μl of bacterial (or spore) suspension (10<sup>8</sup> CFU/ml). Then the inoculum was covered with a sterile polyethylene film and incubated at 37°C for 24 h. After that, the inoculum was transferred into a sterile tube and diluted to 100 ml with sterilized phosphate-buffered saline. Bacterial suspensions were subjected to serial dilution and 100 μl aliquots of each dilution were homogeneously plated on Luria-Bertani (LB) agar (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l). The LB agar plate was incubated at 37°C for 48 h and the bacterial colonies were counted to evaluate antimicrobial performance. For the control, the inoculum was pipette onto films without CEO/β-CD with the other conditions kept constant. Antifungal activity was conducted by the same assay but Sabouraud dextrose agar was

used instead of LB agar. The data are expressed as log reductions relative to control.

### Statistical analysis

Experiments were performed in triplicates; the analysis of variance (ANOVA) and duncan's test were conducted using IBM SPSS statistics 20 software. The results were considered significant at *p* < 0.05.

## Results

### Isolation and identification of CGTase-producing bacteria

Screening of mangrove sediment samples collected from Abu Minqar Island resulted in the isolation of 16 bacterial strains showing yellowish colored zones around their growth, indicating the potential of CGTase production (Fig. S1). The presumptive CGTase-producing isolates were subjected to quantitative determination of CGTase activity. For this purpose, enzyme assay was conducted and results revealed that the enzyme was produced in extracellular fractions of all investigated strains with specific activities ranged from 0.2 to 78.4 U/mg protein. Consequently, five promising isolates designated AMI-1, AMI-2, AMI-3, AMI-4, and AMI-5 were selected and identified by MALDI-TOF/MS. Of these five strains, only four were successfully identified by MALDI-TOF/MS analysis. Further identification was confirmed by 16S rRNA gene amplification and sequencing and results revealed that CGTase-producing isolates are belonging to five different genera (Table 2). Near full-length 16S rRNA gene sequences (1,410–1,425 bp) were obtained and deposited in the GenBank under the accession numbers MK184206 (*Bacillus circulans* AMI-1), MH938812 (*Brevibacillus brevis* AMI-2), MK184207 (*Paenibacillus macerans* AMI-3), MK184208 (*Vibrio parahaemolyticus* AMI-4), and MK184209 (*Oceanobacillus*

**Table 2. Identification and characterization of CGTase-producing bacteria.**

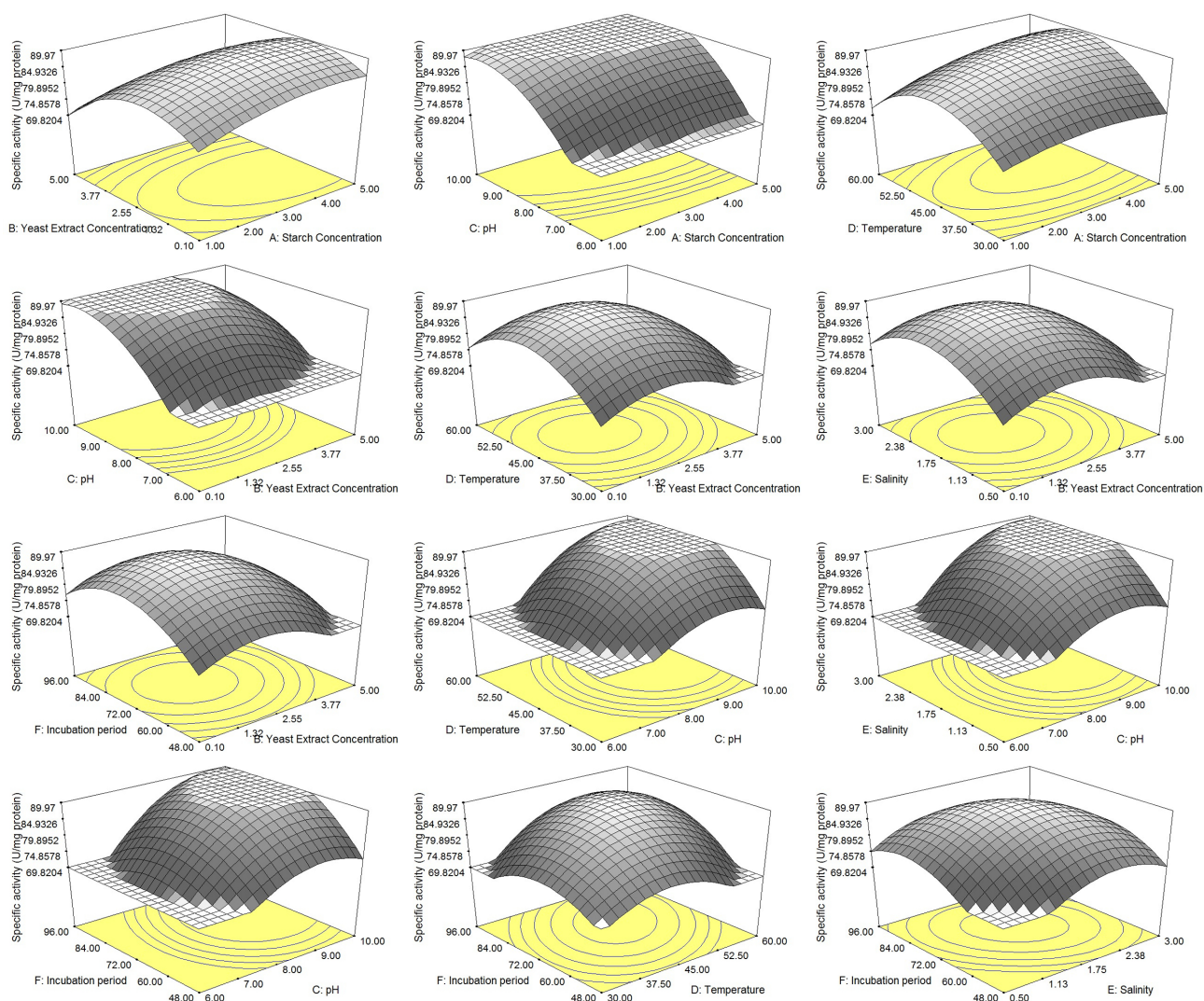
Isolate	MALDI-TOF/MS	16S rRNA	Amplicon size (bp)	Accession number	CGTase activity (U/mg)
AMI-1	<i>Bacillus circulans</i>	<i>B. circulans</i>	1,420	MK184206	8.6 ± 0.7
AMI-2	<i>Brevibacillus brevis</i>	<i>B. brevis</i>	1,410	MH938812	78.4 ± 2.8
AMI-3	<i>Paenibacillus macerans</i>	<i>P. macerans</i>	1,424	MK184207	12.2 ± 0.9
AMI-4	<i>Vibrio parahaemolyticus</i>	<i>V. parahaemolyticus</i>	1,415	MK184208	1.1 ± 1.3
AMI-5	Not identified	<i>Oceanobacillus picturæ</i>	1,421	MK184209	4.8 ± 0.36

*picturae* AMI-5). Based on the results of the quantitative determination of CGTase activity, *B. brevis* strain AMI-2 manifested superior activity was selected for further investigations. A phylogenetic tree of *B. brevis* strain AMI-2 and the most related species was constructed (Fig. S2).

### Optimization of CGTase production using RSM

The combined effect of various cultural variables on CGTase production by *B. brevis* strain AMI-2 was evaluated using RSM to predict the optimized condition for maximum CGTase production. Results obtained from 90 experiments were analyzed using Design Expert software to predict the optimum conditions (Table S1). The

three-dimensional response surface curves were plotted to clarify the interaction of independent variables and to determine the optimum condition (Fig. 1). The optimal levels of a predicted desired activity (98.99 U/mg) were as follows: starch concentration 4.43%, yeast extract 1.67 g/l, initial pH 9.75, salinity 2.25%, incubation period 81.24 h, and incubation temperature 52.77°C. Accordingly, an experiment under the optimal condition predicted by the model was conducted to validate the statistical model. The actual (experimental) values were found to be very close to the predicted values. The predicted response for CGTase production was 98.99 U/mg protein, whilst the actual response was 97.26 U/mg protein.



**Fig. 1. Three-dimensional response surfaces plots showing the effect of interaction various factors on CGTase production.**

**Table 3. Partial purification of CGTase by starch adsorption method.** The same letter in each column indicates no significant difference according to Duncan's multiple range test ( $p < 0.05$ ). Symbol:  $\pm$  represents standard deviation.

Step	Specific activity (U/mg)	Purification (Folds)
Culture supernatant	97.2 ( $\pm 6.2$ ) <sup>b</sup>	-
Partially purified enzyme	2104 ( $\pm 25.2$ ) <sup>a</sup>	21.7 ( $\pm 1.6$ )

### Purification and immobilization of CGTase

The CGTase from *B. brevis* strain AMI-2 was purified by starch adsorption method where the crude enzyme was adsorbed to corn starch and followed by elution of the adsorbed enzyme using β-CD solution. Accordingly, the enzyme was purified at a single step up to 21.69 fold yielding 2104 U/mg protein (Table 3). Moreover, the purified enzyme was immobilized into calcium alginate microspheres and the operational stability of the immobilized enzyme was evaluated in successive cycles of recurring use of the enzyme. The immobilized enzyme strikingly retained up to 87% of its initial activity after 20 cycles of reaction.

### Cyclodextrin production and product specificity of CGTase

In order to specify the glucanotransferase reaction product and determine the conversion ratio, the immobilized CGTase from *B. brevis* strain AMI-2 was incubated with 10% (w/v) soluble starch as a substrate at 50°C for 4 h, subsequently, the reaction mixture was analyzed by HPTLC that unveiled the production of 0.69% α-CD, 91.25% β-CD, and 8.04% γ-CD. Chiefly, CGTase formed β-CD with a small amount of γ-CD and a negligible quantity of α-CD, notably the yield was 51.18, 4.43, and 0.39 g/l, respectively (Table 4). To benefit the advantage of its encapsulation ability, the produced β-CD was purified from the other CDs and crystallized.

**Table 4. Types and ratio of cyclodextrin produced by immobilized CGTase.** The same letter in each column indicates no significant difference according to Duncan's multiple range test ( $p < 0.05$ ). Symbol:  $\pm$  represents standard deviation.

Cyclodextrin type	Production (g/l)	Conversion (%)
α-CD	0.39 ( $\pm 0.06$ ) <sup>c</sup>	0.69 ( $\pm 0.09$ ) <sup>c</sup>
β-CD	51.18 ( $\pm 5.98$ ) <sup>a</sup>	91.25 ( $\pm 2.09$ ) <sup>a</sup>
γ-CD	4.43 ( $\pm 0.70$ ) <sup>b</sup>	8.04 ( $\pm 2.10$ ) <sup>b</sup>

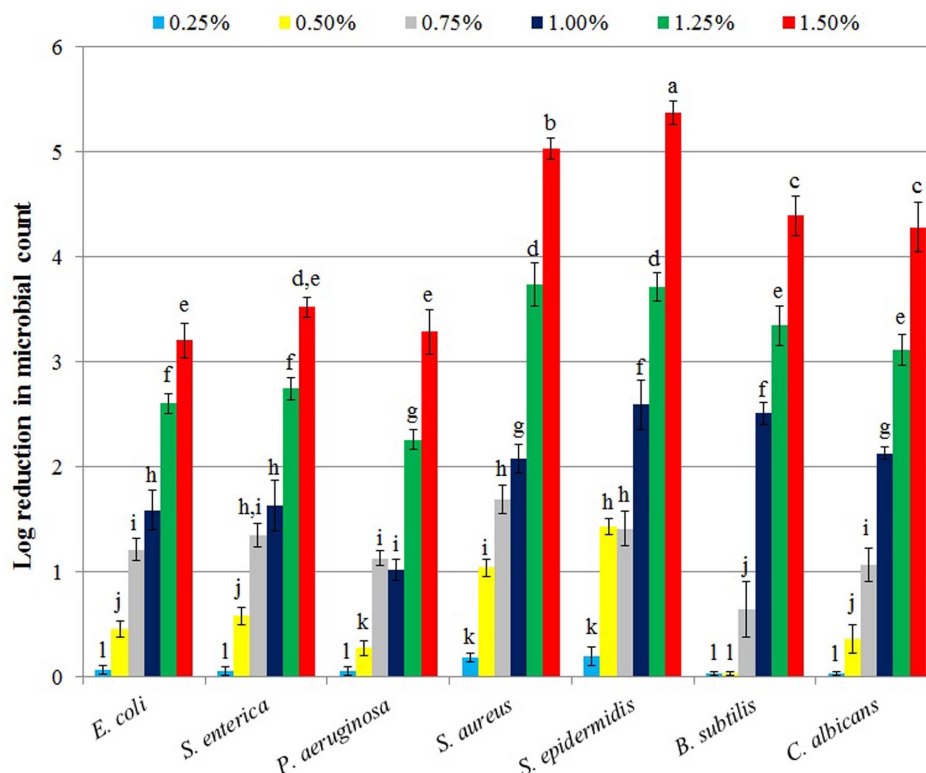
Furthermore, the purified β-CD was used for the preparation of inclusion complex with clove essential oil.

### Preparation of antimicrobial CEO/β-CD-Chitosan film

Essential oil was extracted from clove by steam distillation method and encapsulated in the purified β-CD using co-precipitation method. Subsequently, the produced CEO/β-CD complex was incorporated into a newly fabricated edible chitosan film and antimicrobial activity was appraised. The fabricated CEO/β-CD-chitosan films exhibited a significant antimicrobial activity against all investigated strains particularly *S. aureus* subsp. *aureus* and *S. epidermidis* (Fig. 2). The survival of assessed microorganisms decreased with the increase of CEO/β-CD concentration, which clarifies the antimicrobial activity of CEO/β-CD. The developed CEO/β-CD-chitosan film exhibited the greatest inhibition effect when used at 1.5% concentration against *S. epidermidis* and *S. aureus*, respectively, their count was reduced by more than 5 log CFU. No significant antimicrobial activity was observed against *B. subtilis* spores by using CEO/β-CD at concentrations less than 0.75%, however, 4.3 log reduction was achieved at 1.5% CEO/β-CD. The Log reduction of *E. coli*, *S. enterica* subsp. *enterica*, and *P. aeruginosa* ranged from 3.2 to 3.5 in case of using chitosan film supplemented with 1.5% CEO/β-CD indicating a relatively poor antimicrobial activity against the investigated Gram-negative bacteria. Besides, chitosan film supplemented with 1.5% CEO/β-CD also exhibited a significant antimicrobial activity against *C. albicans* causing 4.28 log reduction in CFUs.

## Discussion

Antimicrobial edible films are presented as an emergent technology capable of increasing the safety and shelf-life of food products. The present investigation addressed the efficient bioconversion of starch into β-CD by β-CGTase derived from *B. brevis* strain AMI-2 and fabrication of edible CEO/β-CD-chitosan films that exhibited a promising antimicrobial activity. In this context, 15 CGTase-producing bacterial strains were isolated from mangrove sediments. It has been suggested that mangrove is a highly productive ecosystem with great microbial diversity [45, 46]. Even though MALDI-TOF is recommended as a rapid microbial iden-



**Fig. 2. Antimicrobial assessment of CEO/β-CD-Chitosan film against various microorganisms.** Columns headed by the same letter were not significantly different according to Duncan's multiple range test ( $p < 0.05$ ). Data were expressed as mean  $\pm$  standard deviations

tification technique due to its accuracy and low-cost compared to other microbial identification methods [47, 48], the present study revealed that 16S rRNA gene analysis is more discriminative compared with MALDI-TOF method. Where *O. picturæ* strain AMI-5 was misidentified by MALDI-TOF method; this limitation may be due to the absence of reference spectra of many marine and environmental bacteria in the database. Similar findings regarding the identification of marine bacteria by MALDI-TOF were reported [49]. According to quantitative screening, 5 potent CGTase-producing strains were selected and identified as *B. circulans* AMI-1, *B. brevis* AMI-2, *P. macerans* AMI-3, *V. parahaemolyticus* AMI-4, and *O. picturæ* AMI-5. As far as the author knows, there are no published data on CGTase production by *Vibrio* and *Oceanobacillus* species. On the other hand, CGTase production from various species belonging to *Bacillus*, and *Paenibacillus* has been extensively described [50–53]. Herein, *B. brevis* AMI-2 was selected owing to its admirable CGTase activity compared with

other investigated strains and subjected to optimization studies. It was noteworthy that RSM optimization enhanced the enzyme production (about 1.24-folds) from 78.4 U/mg to 97.2 U/mg with good correlation between predicted and experimental results confirming the validation of the response model. The statistical optimization indicated the improved CGTase productivity in the presence of relatively high concentrations of NaCl (2.25%) under alkaline conditions (pH 9.75) and elevated temperature (52.77°C). It is assumed that marine bacteria, since they live in the sea, are salt-tolerant organisms and their activities have adapted to the presence of NaCl. Moreover, many reports are available on produced of CGTases from numerous alkalotolerant and alkalophilic strains [51, 54, 55]. Nowadays, RSM is widely reported as an effective statistical technique for designing experiments, building models, evaluating the interactive effects of variables, and pointing the optimum conditions to improve the complex biological processes, especially enzyme production [56–59]. Furthermore, the



enzyme produced under optimum conditions was purified to more than 20-fold by starch adsorption method. These results agreed with several studies whose utilized starch adsorption method as an efficient and inexpensive technique to purify various CGTases [60–62]. Also, alginate-immobilized CGTase exhibited a feasible activity and retained up to 87% of its initial activity after 20 cycles of batching. Immobilization has been realized as a powerful tool to improve the stability, and activity of CGTase by using relatively expensive materials such as chitosan, and silica [63, 64]. In agreement, silica microspheres-immobilized CGTase from *Thermoanaerobacter* sp. retained 60% of its initial catalytic activity after 15 cycles of repeated batches showing its good chemical and mechanical resistance [65] while it maintained 61% of its initial activity after 100 cycles of batch by immobilization on chitosan [63]. In this investigation, the immobilized CGTase converted up to 56% of starch into CDs after 4 h incubation at 50°C. In a previous study, CGTase derived from *B. circulans* ATCC 21783 converted 41% of starch to CDs [60]. Results clarified that  $\beta$ -CD was the major product of CGTase derived from *B. brevis* AMI-2 where the bioconversion profile was 0.69%  $\alpha$ -CD, 91.25%  $\beta$ -CD, and 8.2%  $\gamma$ -CD. The same profile was reported for CGTase from *B. brevis* CD162 but with a relatively different ratio that was 1.1% for  $\alpha$ -CD, 33.2% for  $\beta$ -CD, and 9.9% for  $\gamma$ -CD [66]. It has been suggested that the ratio of the produced CDs is not only dependent on the source of CGTase, but also on the reaction conditions, such as temperature, incubation time, and substrate concentration. Immobilized CGTase from *P. macerans* ACTT 8244 converted 44% of the soluble starch to CDs and the production profile was 79%  $\alpha$ -CD, 16%  $\beta$ -CD, and 5%  $\gamma$ -CD after 6 h but significant change was in the products was observed (43%  $\alpha$ -CD, 56%  $\beta$ -CD, and 1%  $\gamma$ -CD) after 24 h incubation [53]. CGTases is classified into three different types,  $\alpha$ -CGTase,  $\beta$ -CGTase, and  $\gamma$ -CGTase according to the major CD product [67], thus CGTase derived from *B. brevis* AMI-2 is classified as  $\beta$ -CGTase. In this respect, the primary product of starch bioconversion ( $\beta$ -CD) was purified and crystalized yielding 51.18 g/l. In this context, it is worthwhile to exploit the merit encapsulation efficiency of  $\beta$ -CD towards the essential oils. Thus, CEO was extracted and encapsulated in  $\beta$ -CD forming CEO/ $\beta$ -CD inclusion complex. Widely accepted, plant essential oils are good antimicrobial compounds and capable of

preventing the growth of foodborne bacteria [68–70]. A recent study regarding the antimicrobial activity of CEO indicated the superior effect of encapsulation of the essential oil compared to free counterparts [71]. Previous studies proved the improving of antioxidant activity along with enhancing water solubility and high thermal stability of  $\beta$ -CD-encapsulated essential oils and overcoming the restrictions related to their volatility, oxidation and poor water solubility [72, 73]. Apparently, the edible CEO/ $\beta$ -CD-chitosan film addressed here exhibited potent antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as yeast. However, a preeminent antimicrobial activity was observed against Gram-positive compared to that against Gram-negative bacteria and yeast. Generally, Gram-negative bacteria were more resistant to various essential oils than Gram-positive which may be due to the characteristic difference of the outer membrane between Gram-positive bacteria and Gram-negative bacteria [27, 74, 75]. It has been suggested that the hydrophilic cell wall structure of Gram-negative bacteria, constituted essentially by a lipopolysaccharide, blocks the penetration of hydrophobic components of EOs rendering Gram-negative bacteria more resistant toward EOs than Gram-negative bacteria [25]. These findings are in good harmony with several reports concerning the enhanced antimicrobial activity of various essential oils coupled with  $\beta$ -CD [26, 76–78]. Edible films are one of the technologies through which many natural substances could be utilized to extend shelf life on many fresh food products. Therefore, it is highlighted the potential application of the CEO/ $\beta$ -CD-chitosan edible films as antimicrobial agents to assure food safety and quality. The present findings shed light on the potentiality of *B. brevis* AMI-2 as a promising source for the production of CGTase under optimized conditions to synthesize  $\beta$ -cyclodextrin with considerable encapsulation efficiency. Moreover, the obtained results suggest the application of chitosan films in which clove oil is encapsulated in  $\beta$ -cyclodextrin as an edible antimicrobial food-packaging material to combat microbial contamination.

### Conflict of Interest

The author has no financial conflicts of interest to declare.

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