UNIVERSITI PUTRA MALAYSIA

MICROALGAE STRAINS SELECTION AND MEDIUM CONSTITUENTS OPTIMIZATION TO ENHANCE CALCIUM CARBONATE BIOMINERAL PRECIPITATION BY Chlorella vulgaris AND Synechocystis sp. ATCC 27178

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Rapid urbanisation has led to accelerated consumption of concrete. Portland Cement, a key binder in concrete is the most used human-made materials contributing to anthropogenic CO_2 emission. Alternatively, microbially-mediated construction processes and materials could pave ways to more sustainable routes based on the biomineralization process. Precipitation of mineral carbonates by certain microorganisms' metabolic activities can improve the behaviour of concrete or create new construction material. In this study, the potential of eight microalgae strains to undergo calcium carbonate ($CaCO_3$) precipitation to produce cementitious biomineral were assessed, in a process commonly termed as microbially-induced calcium carbonate precipitation (MICP). Initially, these microalgae were cultivated in a medium containing 12 mM $CaCl_2.2H_2O$ and 0.18 to 5.0 mM $NaHCO_3$ and measured for pH, cell growth, calcium concentrations and total alkalinity. *Chlorella vulgaris* and *Synechocystis sp.* ATCC 27178 registered the highest apparent precipitation rate at 0.7 and 0.4 mM/day, respectively, in 5.0 mM $NaHCO_3$ medium. Morphological examination of $CaCO_3$ deposit by SEM-EDX and XRD confirmed it as calcite crystalline structure. These strains were also screened for urease, which catabolises urea as the additional substrate for cell growth and carbonate source for MICP. Consequently, strains having urease activity were cultured in BG-11 medium fixed with 12 mM $CaCl_2.2H_2O$ and 5 mM $NaHCO_3$ but at varying urea concentrations (0 to 0.4 g/L) to investigate urea's effect on $CaCO_3$ precipitation. Carbonic anhydrase and urease activity were assayed, of which, *C. vulgaris* produced the highest precipitation at 0.30 g/L (in 0.2 g/L urea-containing medium) with highest specific urease (SU) activity of 0.127 U/mg/min (on day 2). *Synechocystis* sp. produced 0.411 g/L of $CaCO_3$ (in 0.15 g/L urea-containing medium) with the highest SU of 0.317 U/mg/min (also on day 2). Enhancement to the modified BG-11 (with 12 mM $CaCl_2.2H_2O$ and 5 mM $NaHCO_3$) with urea at 0.2 g/L (*C. vulgaris*) and 0.15 g/L (*Synechocystis* sp.) was achieved through Plackett-Burman Design (PBD), followed by Steepest Ascend Method to search for an effective range, and optimised by Response Surface Method (RSM). PBD screening indicated three significant variables,

i.e., $NaNO_3$, $NaCH_3COO$ and K_2HPO_4 and two positive variables: $NaNO_3$ and $NaCH_3COO$, affecting the response in *C. vulgaris* and *Synechocystis* sp., respectively. Validating the prediction by RSM, modified BG-11 medium optimized with $NaCH_3COO$ (39.5 mM), K_2HPO_4 (0.32 mM) and $NaNO_3$ (19.25 mM) exhibited a productivity of $CaCO_3$ precipitation at 81.6 mg/L/day. It was a 279% improvement over *C. vulgaris* cultivation using modified BG-11 medium fixed with 12 mM of $CaCl_2.2H_2O$, 5 mM of $NaHCO_3$ and 0.2 g/L of urea. For *Synechocystis* sp., by setting $NaCH_3COO$ (60.04 mM) and $NaNO_3$ (0.57 mM), this led to the productivity of 83 mg/L/day. It was 183% more improvement against *Synechocystis* sp. cultivated under identical pre-optimized modified BG-11 medium conditions as *C. vulgaris* but with 0.15 g/L of urea.

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMILIHAN STRAIN MIKROALGA DAN PENGOPTIMUMAN UNSUR MEDIA UNTUK PENINGKATAN PEMENDAKAN BIOMINERAL KALSIUM KARBONAT OLEH *Chlorella vulgaris* DAN *Synechocystis* sp. ATCC 27178.

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Pembandaran yang pesat menyebabkan penggunaan konkrit secara meluas. Simen Portland merupakan bahan asas bagi konkrit dan juga menyumbang kepada pelepasan CO_2 antropogenik. Oleh itu, proses dan bahan pembinaan yang berasaskan mikrob melalui proses biomineralisasi dapat menjurus kepada haluan pembinaan yang lebih baik. Pembentukkan mineral karbonat oleh aktiviti metabolik mikroorganisma dapat mengukuhkan kualiti konkrit dan menjurus kepada penciptaan bahan binaan baru. Kajian ini menguji potensi lapan strain mikroalga dalam pembentukkan biomineral simen iaitu kalsium karbonat ($CaCO_3$) dan proses tersebut dikenali sebagai pemendakan kalsium karbonat berasaskan mikrob. Mikroalga berkenaan dikultur dalam media yang mengandungi 12 mM $CaCl_2.2H_2O$ dan 0.18 hingga 5.0 mM $NaHCO_3$. Nilai pH, pertumbuhan sel, konsentrasi kalsium dan jumlah kealkalian turut diukur. *Chlorella vulgaris* dan *Synechocystis* sp. ATCC 27178 didapati mencatat kadar pemendakan yang tertinggi, iaitu 0.7 dan 0.4 mM/hari dalam media yang mengandungi 5.0 mM $NaHCO_3$. Pemeriksaan morfologi ke atas deposit $CaCO_3$ melalui SEM-EDX dan XRD mengesahkan pembentukkan struktur kristal kalsit. Mikroalga juga disaring untuk enzim urease, yang meghidrolisasikan urea sebagai substrat tambahan bagi pertumbuhan sel dan sumber karbonat. Strain yang mempunyai enzim urease dikultur dalam media BG-11 yang mengandungi 12 mM $CaCl_2.2H_2O$, 5.0 mM $NaHCO_3$, dan 0 hingga 0.4 g/L urea untuk mengkaji kesan urea terhadap pemendakan $CaCO_3$. Aktiviti enzim karbonik anhidrase dan urease turut diuji. *C. vulgaris* didapati menghasilkan pemendakan sebanyak 0.301 g/L $CaCO_3$ (dalam media yang mengandungi 0.2g/L urea) dengan aktiviti spesifik urease (SU) tertinggi iaitu 0.127 U/mg/min (pada hari ke-2). *Synechocystis* sp. pula menghasilkan 0.411 g/L $CaCO_3$ (dalam media yang mengandungi 0.15g/L urea) dengan nilai SU yang tertinggi iaitu 0.317 U/mg/min (pada hari ke-2). Seterusnya, penambahbaikan terhadap media BG-11 yang mengandungi 12 mM $CaCl_2.2H_2O$, 5 mM $NaHCO_3$ dan 0.2 g/L urea (*C. vulgaris*) dan 0.15 g/L urea (*Synechocystis* sp.) dicapai melalui Rekabentuk Plackett-Burman (PBD), diikuti dengan Kaedah Pendakian Kecuraman dan Kaedah Permukaan Sambutan (RSM). Hasil

penyaringan PBD mendapati $NaNO_3$, $NaCH_3COO$ dan K_2HPO_4 mempengaruhi tindakbalas ke atas *C. vulgaris* manakala, $NaNO_3$ dan $NaCH_3COO$ mempengaruhi tindakbalas ke atas *Synechocystis* sp. Bagi mengesahkan hasil RSM, media BG-11 diubahsuai lagi dengan 39.5 mM $NaCH_3COO$, 0.322 mM K_2HPO_4 dan 19.25 mM $NaNO_3$. Hasil mencatatkan produktiviti pemendakan $CaCO_3$ pada 81.6 mg/L/hari dan peningkatan sebanyak 279% berbanding kultur *C. vulgaris* dalam media BG-11 yang mengandungi 12 mM $CaCl_2.2H_2O$, 5 mM $NaHCO_3$ dan 0.2 g/L urea. Manakala bagi *Synechocystis* sp., 60.04 mM $NaCH_3COO$ dan 0.57 mM $NaNO_3$ menghasilkan produktiviti sebanyak 83 mg/L/hari. Ia menunjukkan peningkatan sebanyak 183% berbanding *Synechocystis* sp. yang dikultur dengan media BG-11 yang menyamai keadaan media *C. vulgaris* tetapi dengan 0.15 g/L urea.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AH	Alkaline Hypochlorite
ATCC	American Type Culture Collection
BCM	Biologically Controlled Mineralization
BG-11	Blue Green 11
BIM	Biologically Induced Mineralization
BSA	Bovine Serum Albumin
CA	Carbonic Anhydrase
CCD	Central Composite design
CCM	Carbon Dioxide Concentrating Mechanism
Ca^{2+}	Calcium Ion
$CaCl_2.2H_2O$	Calcium Chloride Dihydrate
$CaCO_3$	Calcium Carbonate
DIC	Dissolve Inorganic Carbon
DCW	Dry Cell Weight
dt	Doubling Time
EBT	Eriochrome Black T
EPS	Extracellular Polymeric Substance
HCO_3^-	Bicarbonate Ion
K_2HPO_4	Dipotassium Phosphate
KH_2PO_4	Potassium Dihydrogen Phosphate
meq/L	Miliequivalent per liter
MICP	Microbially Induced Calcium Carbonate Precipitation
mM	Milimolar

Na_2CO_3	Sodium Carbonate
$NaCH_3COO$	Sodium Acetate
$NaHCO_3$	Sodium Bicarbonate
NH_3	Ammonia
NH_4Cl	Ammonium Chloride
NH_4^+	Ammonium Ion
OD	Optical Density
OH^-	Hydroxyl ion
PN	Phenol Nitroprusside solution
R_2	R-square
PBD	Plackett-Burman Design
pNPA	p-Nitrophenyl Acetate
pNP	p-Nitrophenol
SEM-EDX	Scanning Electron Microscopy with Energy Dispersive X-Ray
SI	Saturation Index
Sp.	Species (singular)
TAP	Tris-base Acetate Phosphate
U/mg	Micromol per milligram
XRD	X-Ray Diffraction
Xmax	Final cell concentration
Xo	Initial cell concentration
μmax	Maximum specific growth rate

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The rapid development of modern societies has led to a drastic impact on global warming, and this reality is none more so exemplified than in the construction sector. As much as it is crucial to driving a nation's economy, its major activity nonetheless inevitably contributes to the liberation of Green House Gases (GHG) through the massive consumption of concrete. Cement is an essential key binder ingredient of concrete used predominantly due to its relatively low cost and high strength (Ivanov *et al*., 2015). It is used widely for the construction work of roads, buildings and sealing of cracks on existing historical buildings for conservation purposes. Demand-wise, about 3.7 – 4.4 billion tons of production materials are expected to be consumed by 2050 (Lee *et al.*, 2018). The transformation process of cement through the decarbonization of natural limestone is very energy-intensive. During production, the calcination of 1-ton limestones will emancipate approximately 0.814 tons of carbon dioxide (CO_2), accounting for 6-8% of global anthropogenic CO_2 emissions (Choi *et al.*, 2017; Ivanov *et al.*, 2015).

For this reason, scientific development in sustainable environmental initiatives and low-cost biomaterials for construction uses or other multidisciplinary fields are requisites to mitigate CO_2 emissions. Microbially-mediated construction processes utilizing biomineralization pathways are among the top initiatives to curb this problem. Many researchers have explored the possibilities of manipulating the "Microbially Induced Calcium Carbonate Precipitation" (MICP) metabolic process in microorganisms that could yield biocement material as a viable alternative to conventional cement (Gleaton *et al.*, 2019; Irfan *et al.*, 2019). The novel idea of employing MICP is reliable as the phenomenon is quite common in nature and manifested in many bacteria, algae, and fungi, albeit occurring very slowly over long geological times for certain microorganisms. The criteria of microorganisms to produce calcium carbonate ($CaCO_3$), a cementitious biomineral, include the effect of physicochemical aspects such as pH, nucleation site for precipitation, concentration of calcium, and concentration of dissolved inorganic carbon (DIC), urease and carbonic anhydrase activity (Ariyanti *et al*., 2012). Besides that, the formulation of growth media of microorganisms is important in increasing the production of biocement.

Biocement can bind porous materials together and reinforce their mechanical properties, such as strength and impermeability (Omoregie *et al*., 2019). This natural binder can be added into a specialized matrix for soil stabilization or serves as an exterior material of a building to remediate cracks, pores or voids on which it provides adequate strength within a month (Choi *et al.*, 2017; Lee *et al.*, 2018; Seifan *et al.*, 2018). MICP process can be divided into Biologically Induced Mineralization (BIM) and Biologically Controlled Mineralization (BCM). BIM occurs when the minerals are formed due to the microorganism's metabolic activity, whereas in BCM, mineral formation is entirely governed by the microorganisms' cellular activity and directly synthesized at a specific location (Saad *et al.*, 2018). MICP process employs many established pathways such as photosynthesis, dissimilatory sulphate reduction, ammonification of amino acids, denitrification, and urea hydrolysis. These pathways produce inorganic $CaCO_3$ as an end-product which will be transformed as a potential biocement (Anbu *et al.*, 2016; Anitha *et al.*, 2018).

To date, much of the research on biocement development place their focus on ureolytic bacteria such as *Sporosarcina pasteurii*, *Bacillus pasteurii*, and *Brevundimonas* sp., which utilizes urea as a potential substrate to produce additional carbonate source and favours the production of inorganic $CaCO_3$ precipitation (Choi *et al.*, 2017; Wei *et al.*, 2015). Regardless, the utilization of bacteria still has a few disadvantages, such as the generation of toxic by-products, unpleasant odour and expensive treatments. This leaves a gap in fulfilling the needs for sustainable construction materials and environmentally friendly processes. For the past few decades, microalgae and cyanobacteria have shown enormous potential in a wide breadth of applications such as soil remediation, CO_2 sequestration, removal of heavy metals and radionuclides contaminants, but exploratory works on biocement are still confined to the laboratory working stage, and biotreatments trials in structural applications are yet to be tested. Few studies in the laboratory stage have unravelled the biomineralization activity of microalgae and cyanobacteria such as *Chlorella kessleri*, *Mychonastes* sp., *Nannochloropsis* sp., *Gloeocapsa* sp., *Synechococcus* sp., *Thraustochytrium striatum* and *Scytonema* sp. occurring mainly through photosynthesis pathway and has a great potential to be used as biocement that comparable to bacteria (Bundeleva *et al*., 2014; Gleaton *et al*., 2019; Irfan *et al*., 2019; Zhu *et al.*, 2015, 2018). The primary role of microalgae in carbonate precipitation is their ability to create an alkaline environment through their various physiological activities, including photosynthesis, urease and carbonic anhydrase activities. Photosynthesis plays a significant role by increasing DIC availability in the immediate habitats as the CO_2 consumed is subsequently converted into a carbonate source in the metabolic pathway of microalgae.

Similarly, an increase in urease activity will lead to hydrolysis of urea which enhances the precipitation of $CaCO_3$ by providing additional carbonate sources (Peng & Liu, 2019). The presence of carbonic anhydrase intracellularly and extracellularly facilitates the conversion of CO_2 to bicarbonate ion (HCO_3^-) or vice versa, leading to an increase in the alkaline pH of the medium and facilitating $CaCO_3$ precipitation (Zawar *et al*., 2016). $CaCO_3$ precipitate is permanently removed as a solid in various polymorphs depending on its growth condition. It may form three anhydrous polymorphs: calcite, aragonite, vaterite, or two hydrated crystalline phases: monohydrocalcite and ikaite (Krajewska, 2018). Calcite is usually considered the most stable and least soluble polymorph, appropriating for biocement applications (Bundeleva *et al*., 2014; Zhu *et al*., 2018). Besides that, electronegative cell surface and exo-polymeric substances (EPS) serve as a nucleation site, and a high degree of saturation state due to metabolic changes surrounding the cell facilitates the formation of $CaCO_3$ (Zawar *et al*., 2016). Moreover, the medium formulation is equally important in the mixotrophic cultivation of microalgae to enhance the growth and provide additional nucleation sites for precipitation of $CaCO_3$. The medium composition must fulfil all necessities for cell growth and metabolite production by providing sufficient energy for biosynthesis and cell maintenance (Chin *et al.*, 2020). Therefore, major attention is needed to optimize medium composition to maximize microalgal biomass and MICP activity.

1.2 Problem Statements

Portland Cement is more popular among concrete producers as it has been used since the 18th century, and the acceptance of biological processed material in the construction community is still low. This leaves a gap in fulfilling the needs for sustainable construction materials and environmentally friendly processes. The exploratory works on the potential of local microalgae as a source of $CaCO_3$ for biocement material have been limited to the laboratory working stage. Besides that, the cultivation of calcification experiments on potential microalgae strains is confined to small scall development in a sterile environment. The occurrence of any cross-contamination may affect the biomineralization activity of microalgae strains.

1.3 Scope of the Study

This study determined that the microalgae strains- available in the local depository could potentially induce $CaCO_3$ biomineral precipitation. The assessment primarily considered the microalgal cellular growth and biomineralization capacity as they were affected by varying bicarbonate concentrations. For strains indicating positive $CaCO_3$ deposition, the resulting biomineral would be further characterized through Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (SEM-EDX) and X-ray Diffractometry (XRD) analyses. Following that, the biomineralization activity of the best MICP microalgae species was examined based on their ability to grow in a mixotrophic condition. The optimization of medium composition was done using statistical methods. The selected medium compositions and parameters were based on the literature review, and they would be screened for their significance towards the productivity of $CaCO_3$ via Plackett-Burman Design. The identified significant factors were optimized via the steepest ascent method and, lastly, through the Central Composite Design to determine the optimal level of maximized biogenic $CaCO_3$.

1.4 Objective of This Study

1. To determine the highest capacity of biomineralization of calcium carbonate precipitates by microalgae strains obtained from local depositories.
2. To investigate the effect of urea concentrations and physicochemical parameters on the kinetic urease and carbonic anhydrase activity of the selected MICP microalgae.
3. To optimize the significant factors affecting the productivity of $CaCO_3$ precipitation by the selected MICP microalgae using statistical methods of Plackett-Burman Design (PBD) and Response Surface Methodology (RSM).

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