UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND OPTIMIZATION OF BIOFLOCCULANT DERIVED THROUGH IMPLEMENTATION OF SUBMERGED AND SOLID-STATE FERMENTATION OF Bacillus subtilis UPMB13

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By

ZUFARZAANA ZULKEFLEE

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CHARACTERIZATION AND OPTIMIZATION OF BIOFLOCCULANT DERIVED THROUGH IMPLEMENTATION OF SUBMERGED AND SOLID-STATE FERMENTATION OF *Bacillus subtilis* UPMB13

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October 2014

Chair : Mohd Kamil bin Yusoff, PhD

Faculty : Environmental Studies

Breakthroughs in bioproduction field have opened up vast opportunities in the exploration of bio-based products as substitutes to chemical derivatives for water treatment technologies. In that framework the interest of finding an ecologically benign solution specifically focusing on treating suspended solids pollution was explored. As production cost becomes the limiting factor which restricts wider applications of bioproducts for alternative water treatment, fermentation technology was applied in this study; basic substrates were utilized through non-elaborative techniques for bioproduction of a biopolymeric flocculant. The main goal of this study is to produce a biopolymer with flocculating capabilities which can substitute the commonly used commercial flocculants through two fermentation strategies; namely, the submerged (SmF) and solid-state (SSF) fermentations. The flocculating performances were measured through kaolin assays based on the clarity of the suspension and the visible flocs formed after treatment with the bioflocculant. The characterizations of the bioflocculants produced were scrutinized for further understanding of their nature and properties which contributed to their flocculating abilities. The bioflocculant produced through the better fermentation strategy was further studied for its flocculating performances and in comparison to other commercial flocculants. A novel low molecular weight (10-50 x 10^3 Da), high flocculating biopolymer denoted as UPMBF13 was successfully produced through *de novo* pathway from the SmF and the SSF of *Bacillus subtilis* UPMB13. It was found to consist of poly-γ-glutamic acid and polysaccharide derivatives, with hydroxyl, carboxyl, methoxyl and carbonyl functional groups and was either fibrous (SmF) or granular (SSF) in natures, which are the major known characteristics of a bioflocculant. The best production strategy for UPMBF13 was found to be through the SmF by manipulating the optimum conditions (media: no additional supplement; duration: 24-72 hrs; temperature: 25-30^{o}C; pH: 7.0-8.0; shaking speed: 100-200 rpm) for growth. This led to a maximum performance of 95% in flocculating activity with large visible floc formations, comparable to those from the commercial flocculant polyacrylamide (maximum activity: 98%), and superior to that of polyaluminium chloride (maximum activity: 47%). The production of UPMBF13 through SSF was also verified to be possible, but yielded an inferior product (maximum activity: 71%) with barely any flocs formed upon treatment. Furthermore, the SmF strategy yielded at an average two-fold the amount of UPMBF13 at 2.70 g/L while the SSF produced about 1.25 g/kg in 72 hrs. Overcoming the inferior performance of the SSF by scaling-up the process to a pilot-scale level (near-to-adiabatic, non-sterilized condition with continuous oxygen flow) led to a competitive environment where the autochthonous microbes proliferated over UPMB13 and produced their own bioflocculants which obscured the performance of UPMBF13. In general, the results from this study confirmed that the production of UPMBF13 is feasible through *de novo* pathway with no additional input of L-glutamic acid supplement. High flocculating performance was achieved solely with basic substrates without further manipulations and modifications. Furthermore, UPMBF13 is cation-independent once extracted and purified, requiring no additional cation source for its application in suspended solid treatments.

Keywords: *Bacillus subtilis* UPMB13, bioflocculant, extracellular polymeric substance, biopolymer, submerged fermentation, solid-state fermentation

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN DAN PENGOPTIMUMAN BIOFLOKULAN YANG DIHASILKAN MELALUI IMPLEMENTASI KAEDAH FERMENTASI TERENDAM DAN FERMENTASI KEADAAN PEPEJAL KE ATAS *Bacillus subtilis* UPMB13

Oleh

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Oktober 2014

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Kejayaan cemerlang dalam bidang biopembuatan telah membuka banyak peluang dalam penerokaan produk berasaskan biologi sebagai pengganti kepada derivatif-derivatif kimia dalam teknologi rawatan air. Selaras dengan itu, kepentingan mencari penyelesaian yang lebih mesra alam, khususnya dalam merawat pencemaran pepejal terampai telah diterokai. Oleh kerana kos pengeluaran telah dikenal pasti sebagai faktor pengehad yang menyekat penggunaan bioproduk sebagai rawatan air alternatif secara meluas, teknologi fermentasi telah digunakan dalam kajian ini; substrat asas telah digunakan melalui teknik mudah untuk biopembuatan flokulan berasaskan biopolimer. Matlamat utama kajian ini adalah untuk menghasilkan sejenis biopolimer dengan keupayaan flokulasi yang boleh menggantikan flokulan-flokulan komersial yang biasa digunakan melalui dua strategi fermentasi iaitu kaedah fermentasi terendam (SmF) dan fermentasi keadaan pepejal (SSF). Kadar prestasi flokulasi diukur melalui ujian menggunakan kaolin berdasarkan kejernihan ampaian dan penghasilan flok selepas rawatan menggunakan bioflokulan tersebut. Pencirian bioflokulan yang dihasilkan diteliti untuk pemahaman lebih lanjut tentang sifat-sifat asas yang menyumbang kepada kebolehannya sebagai flokulan. Bioflokulan yang dihasilkan melalui kaedah fermentasi yang lebih baik telah dikaji lebih lanjut bagi mengenalpasti faktor bagi meningkatkan kadar prestasi flokulan dan mengkaji perbandingan prestasi bioflokulan tersebut dengan beberapa flokulan komersial. Sebuah biopolimer novel yang berprestasi tinggi dengan jisim molekular rendah (10-50 x 10^3 Da), dinamakan sebagai UPMBF13 telah berjaya dihasilkan melalui laluan *de novo* dari SmF dan SSF *Bacillus subtilis* UPMB13. Ia didapati terdiri daripada asid poli-γ-glutamik dan derivatif polisakarida dengan kumpulan berfungsi hidroksil, karboksil, metoksil and karbonil serta bersifat samada bergentian (SmF) atau berbutir (SSF), di mana kesemuanya dikenali sebagai ciri-ciri major bioflokulan. Kaedah fermentasi terendam telah dibuktikan sebagai kaedah terbaik bagi pengeluaran UPMBF13 melalui manipulasi pertumbuhan dan penyediaan keadaan optimum (media: tiada supplemen tambahan; tempoh: 24-72 jam; suhu: 25-30^{o}C; pH: 7.0-8.0; kelajuan goncangan: 100-200 rpm). Ini telah membawa kepada kadar prestasi flokulasi maksimum sebanyak 95% dengan pembentukkan flok besar dapat diperhatikan, setanding dengan flokulan komersial poliakrilamida (flokulasi maksimum: 98%) dan lebih tinggi daripada polialuminium klorida (flokulasi maksimum: 47%). Kaedah fermentasi keadaan pepejal juga telah dibuktikan mampu dilaksanakan bagi pengeluaran UPMBF13, namun menghasilkan produk yang lebih rendah prestasi (flokulasi maksimum: 71%) dengan hampir tiada pengahasilan flok. Tambahan pula, kaedah SmF berjaya menghasilkan UPMBF13 pada kuantiti dua kali ganda; iaitu 2.7 g/L, manakala kaedah SSF hanya menghasilkan 1.25 g/kg UPMBF13 dalam masa 72 jam. Bagi mengatasi prestasi rendah kaedah SSF, percubaan menaikkan skala proses SSF di tahap skala perintis (keadaan hampir adiabatik, tidak steril dengan aliran oksigen berterusan) telah meningkatkan persekitaran yang lebih berdaya saing di mana mikroorganisma sedia ada yang hadir dalam substrat telah mengatasi pertumbuhan UPMB13 dan menghasilkan bioflokulan mereka sendiri yang mengatasi prestasi UPMBF13. Secara amnya, hasil kajian mengesahkan pengeluaran UPMBF13 boleh dilaksanakan melalui laluan *de novo* tanpa memerlukan penambahan asid L-glutamik. Kadar prestasi flokulasi yang tinggi telah dicapai dengan hanya menggunakan substrat asas tanpa manipulasi dan pengubahsuaian lanjut. Tambahan lagi, UPMBF13 bebas kebergantungan terhadap kation setelah diekstrak, oleh itu tiada sumber kation tambahan yang diperlukan bagi merawat pencemaran pepejal terampai.

Kata kunci: *Bacillus subtilis* UPMB13, bioflokulan, bahan polimer luar sel, biopolimer, fermentasi terendam, fermentasi keadaan pepejal

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I certify that a Thesis Examination Committee has met on 27 October 2014 to conduct the final examination of Zufarzaana binti Zulkeflee on her thesis entitled "Characterization and Optimization of Bioflocculant derived through Implementation of Submerged and Solid-state Fermentation of *Bacillus subtilis* UPMB13" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

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

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- the research conducted and writing of this thesis was under our supervision;
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ABBA:	Alpha Amino Butyric Acid
ANOVA:	Analysis of Variance
ASTM:	American Society for Testing and Materials
ATR:	Attenuated Total Reflectance
AQC:	6-AminoQuinolyl-N-hydroxysuccinimidyl Carbamate
BLAST:	Basic Local Alignment Search Tool
BME:	β-mercaptoethanol
DNA:	Deoxyribonucleic Acid
DVLO:	Derjaguin, Verwey, Landau and Overbeek theory
EDA:	Exploratory Data Analysis
EPS:	Extracelullar Polymeric Substances
FT-IR:	Fourier Transform Infra-Red
HA:	Hyaluronic Acid
HCl:	Hydrochloric Acid
HPLC:	High Performance Liquid Chromatography
KBr:	Potassium Bromide
LDS:	Lithium Dodecyl Sulfate
LSD:	Least Significant Difference
MWCO:	Molecular Weight Cut-Off
NaOH:	Sodium Hydroxide
OD:	Optical Density
OECD:	Organisation for Economic Co-operation and Development
PAC:	Polyaluminium Chloride

PAM:	Polyacrylamide
PCR:	Polymerase Chain Reaction
PGA:	Poly-glutamic Acid
PHA:	Polyhydroxyalkanoates
RPM:	Revolutions per Minute
rRNA:	Ribosomal Ribonucleic Acid
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide Gel
SEM:	Scanning Electron Microscope
SmF:	Submerged Fermentation
SSF:	Solid-state fermentation
TSA:	Tryptic Soy Agar
TSB:	Tryptic Soy Broth
TSS:	Total Suspended Solids

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

The current trend and advances in the emerging environmentally benign approaches for solving environmental issues bring forth vast opportunities in the exploration of microbial-derived products as promising substitutes to the present dependence on the chemical-based derivatives (Hatti-Kaul *et al.*, 2007). According to a global market review in 2011 and updated in 2013 on the trend of the demand for microbial products, the total global market worth was projected to approach 179 billion U.S Dollars (RM592 billion) by the year 2018 (McWilliams, 2013) with among the highest demand of these biologically-derived products coming from the environment-related industries. These claimed bioproducts of environmental importance are now globally accepted and approved as alternatives to the conventional chemicals that had long been scrutinized for the adverse side effects they pose, not only to the environment but also to human health (OECD, 2011). To name a few, these environmental-friendly bioproducts include biofuels, biosurfactants, biopesticides, biofertilizers, biopolymers and many more that are of interest in the environmental sectors.

Productions of these biologically-derived products are based on the extraordinary capabilities of microorganisms which can be exploited through the manipulation of their genetics, growth and metabolism. Despite the immense prospect of these bioproducts, the apparent barriers that prevent them from being widely employed are implementation costs required in all aspects of their production (Christenson and Sims, 2011). Nevertheless, the exceptional developments in biological sciences nowadays are focused on solving this issue and thus are opening up various opportunities in the research and study of potentially new bioproducts through genetic modifications, fermentation technologies and others (OECD, 2011).

In relation to this current issue, there is a greater demand to seek new eco-friendly means of utilizing naturally-derived bioproducts for application in water treatment processes. The role of water as the basic necessity to mankind defines the need for it to be purified or treated before being consumed or used by humans through various treatments, especially for potable consumption and recreational purposes. One of the important processes in water purification is the solid-liquid separation of the suspended solids present in the water system (Devesa-Rey *et al.*, 2012; Brostow *et al.*, 2009).

The efficiency of water purification could be enhanced through induced aggregation processes (Maximova and Dahl, 2006). Common methods used for the enhancement of the aggregation phenomenon in the treatment of suspended solids in water are the usage of chemical coagulants and flocculants as aids in the agglomeration of the suspended particles into bigger clumps resulting in a higher sedimentation rate by gravitational pull (Brostow *et al.*, 2009). These settled clumps or flocs are then readily available to be collected or filtered by physical means. The usage of these chemical flocculants and coagulants; however efficient they may be, always require higher input costs. Concerns also arise on the harmfulness of their residual derivatives as well as monomers to both the environment and human health (Rudén, 2004; Flaten, 2001). Hence, the imminent need of an environmental-friendly alternative. The production and utilization of a biopolymer, biologically-derived polymer, as a potential flocculant, commonly known as a bioflocculant, was, therefore, explored.

Currently, the use of biological flocculants as substitutes to the common commercial chemical flocculants are being concertedly researched, recognized and accepted. Various sources of biological flocculants are being introduced as environmental-friendly alternatives. The unique characteristics of being naturally produced, biodegradable, and environmentally safe are some of the advantages of these natural products (Deng *et al.*, 2003).

Studies on the production of bioflocculants are widely competitive and rapidly emerging. Though new microbial species are the main focus, the established species are still being exploited since they may still produce specific bioflocculants with different novel characteristics and performances, depending on their fermentation conditions and other introduced factors (Kimura *et al.*, 2013). Thus, researches on the feasibility of different fermentation methods for sustained production of these bioflocculants are greatly encouraged. Another important consideration in bioflocculant research is the production cost, one of the limiting factors in commercial application of bioflocculants. Hence fermentation technology is another vital component to be further explored (Subramanian *et al.*, 2009a). New and more environmentally friendly means and cost-cutting measures in bioproduction processes need to be uncovered to meet the current market demand for biopolymers (Oner, 2013). Thus, the field of bio-processing is currently focused on the development of these processes through non-elaborative techniques with cheaper fermentation substrates.

1.2 Problem Statement

In the Malaysia Environmental Quality Report 2007-2011, suspended solids were reported as one of the major sources of river water pollution in Malaysia, together with Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). Suspended solids were also reviewed to be an imperative cause of water quality dilapidation including ecological degradation of aquatic organisms and environments, aesthetic degradation as well as incurring higher cost in water treatments (Bilotta and Brazier, 2008).

Suspended solids refer, especially but not solely, to soil solids resulting mainly from earthworks, land clearing activities and deforestation together with other associated development activities. Suspended solids can be described literally as solids that remain suspended in water without being readily settleable. They are materials that can be filtered or separated physically or chemically from the water bodies, with the appropriate treatment. They may contain materials such as animal and plant wastes or decays, silts and soil solids. These suspended solids will affect the turbidity in a given water body as well as possible encumbrance on oxygen depletion due to the high organic content and naturally occurring decomposition in the water system (Bilotta and Brazier, 2008).

Over the years, land developments for agriculture such as farming and crop cultivations, and industrial activities, businesses and housing developments have increased sharply due to the high demand of urbanization and population growth. Consequently, extensive land clearing are subjected on forested areas, exposing greater areas to erosion problems. During regular rainfall, the exposed soil surface results in increased surface runoff which leads to the eminent increase in sediment loading into the river system. Thus, the total suspended solids and turbidity become some of the main contributors to the decline in water quality. It is, therefore, crucial to find a solution to this crisis.

Besides this obvious effect of turbidity, another immediate concern regarding the issue of suspended solid pollution is the accumulation of pollutants *viz.* persistent organic pollutants such as pesticide residues, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated bipenyls (PCBs) (Gdaniec-Pietryka *et al.*, 2013; Liu *et al.*, 2013; Bakhtiari *et al.*, 2009), nutrients including phosphates and nitrates (Shinohara *et al.*, 2012; Edwards and Withers, 2008), heavy metals (Zheng *et al.*, 2013), and pharmaceutical by-products and hormones (emerging pollutants) (Rivera-Utrilla *et al.*, 2013; Deblonde *et al.*, 2011; Maskaoui and Zhou, 2010); in the suspended particles. It was reported by Ferreira da Silva *et al.* (2013) that the occurrences of these emerging pollutants in the suspended particles found in surface water are becoming a rising concern due to the incomplete removal of these pollutants during wastewater treatment which could lead to their discharge as ubiquitous components of the effluent into the aquatic environment. Furthermore, as these emerging pollutants are previously non-regulated, strict guidelines are absent to limit their discharge to the environment (Bolong *et al*., 2009). The removal of these emerging pollutants from treated samples is mainly due to the removal of suspended solids in the samples where the pollutants are introduced (Dagnino *et al*., 2010). Hence, the importance of removing suspended solids in the water system.

The current dependence on chemical and synthetic flocculants for the treatment of suspended solids can pose threats to human health and the environment due to the potent carcinogenic and toxic properties of the monomers and non-biodegradable nature of the by-products of these flocculants (Buraimoh and Ojo, 2013; Park *et al.*, 2010; Manafi *et* *al.*, 2010; Kumar and Gill, 2009). A safer alternative is to use biological treatments which are being sought to substitute the usage of these chemicals. They are comparatively more environmentally safe (Muthulakshmi *et al.*, 2013). Furthermore, it was also reported that the flocs produced through the treatments with these synthetic flocculants were less stable and prone to disintegrate from shear force while the natural flocculants produced flocs that were more shear resistance (Brostow *et al.*, 2009); thus, giving them a technical advantage over the synthetic flocculants.

Gamarra *et al.* (2010) reported that the study of bioproduction through submerged fermentations (SmF), solid-state fermentations (SSF) and also biofilm were still lacking and need to be explored. Breakthroughs in fermentation technologies with regard to the manipulation of cultural conditions for bioproduction have compared the potentials of submerged and solid-state fermentations with emphasis on the respective advantages and disadvantages. The products from both methods could be similar (Das and Mukherjee, 2007) or superior (Sandhya *et al.*, 2005) to one another, hence creating a scope of knowledge with various possibilities that need to be fully appreciated for better understanding and management to meet future demand in a bio-based era.

The production of bioproducts have been markedly dominated by the applications of submerged fermentations owing to minimal hassles required in its handling, monitoring, and overall process and control (Singhania *et al*., 2010). It has been proven that bioflocculant productions from various microorganisms are viable through the employment of SmF with future prospects of commercialization. However, concerns do arise on the high cost of the process originating mainly from the cost of the required substrates. Therefore, simpler, non-elaborative techniques are needed to curtail production cost of SmF.

Predominance of bioflocculant production through submerged fermentations has inadvertently overlooked the prospect of solid-state fermentations. SSF are known to be widely implemented for the production of enzymes, biosurfactants and even biopolymers (Thomas *et al.*, 2013) as substitute to the SmF. Though the production of biopolymers through SSF had also been reported, however, there is no documented evidence on the production of bioflocculants through the SSF. Therefore, taking the platform of going bio-based in the production of bioflocculants, the SSF method was also employed on the basis of utilizing organic wastes as substrates. Furthermore, utilization of the SSF will curtail production cost giving an economic advantage as well as creating a solution for the utilization of the abundant agricultural and organic wastes (Dhillon *et al.*, 2013). Nonetheless, the SSF process could be a challenge due to the inherent complexity of managing bulk quantities of solid substrates (Abraham *et al.*, 2013). This raise uncertainty in controlling and standardizing the fermentation process for regular and continuous bioflocculant production in the long run.

Notably, there are some advantages and disadvantages of both methods; thus, the extent of their feasibility and efficiency in bioflocculant production will be evaluated.

1.3 Research Questions

1. Can bioflocculants be produced through submerged and solid-state fermentations?
2. What are the optimal setup for bioflocculant production from both fermentation strategies?
3. Would there be any differences between bioflocculants produced through submerged and solid-state fermentations in terms of yield, characteristics, quality and performances?
4. Can the bioflocculant compete with other commercial flocculants?
5. How would the bioflocculant fair in treating real water samples?

1.4 Objectives

1. To compare the production of the bioflocculant through submerged and solid-state fermentations
2. To extract a potential bioflocculant produced by *Bacillus subtilis* UPMB13
3. To characterize the extracted bioflocculants from both fermentations for further understandings of their nature and capabilities
4. To evaluate the flocculating performances of the selected bioflocculant and to compare its performance with other commercial flocculants

1.5 Significance of Study

This research will address, generally, the current interest of producing biologically-derived products of environmental importance that may substitute present dependence on chemical derivatives as a solution to various environmental issues, especially those related to water pollution. Specifically, this research bring forth the innovation of using the bioflocculant treatment in solving issues of reducing suspended solid pollution through safe and environmentally sound manner.

Indirectly this research aims to contribute to the effort of remediating suspended solids related issues including the problem of turbidity and pollutant accumulations in surface water through the introduction of a novel bioflocculant produced from a locally isolated rhizhobacterium.

By employing two different fermentation strategies, the study will explore the possibilities of bioflocculant production from each fermentation technique creating a basic outlook of the feasibility of this innovation for future prospects of mass production and possible commercialization. This approach would benefit the water treatment industries as well as contribute to the present knowledge on fermentation technologies and bioproduction in the field.

1.6 Thesis structure

The thesis focuses on the production of a biopolymer from the selected bacterium; *Bacillus subtilis* UPMB13 with flocculating capabilities. Issues addressed in the thesis include the factors affecting production of the bioflocculants, characterization of the extracted bioflocculants with comparison of two fermentation strategies selected; namely, submerged and solid-state fermentations and flocculating performances of the chosen bioflocculant in comparison with commercial flocculants.

The following section depicts the flow and outline of the thesis:

Chapter 2 lists the literature reviews of previous researches done in relation with this research. Included within the chapter are the definitions of suspended solids, issues related to suspended solids pollution, flocculants and bioflocculants, principle mechanisms of flocculation, various producers of bioflocculants, description on submerged and solid-state fermentations, factors affecting production and bioflocculation performance, characterization of the bioflocculant and applications of the bioflocculant.

Chapter 3 explains the general materials and methods used all throughout the research focusing on the culturing and maintenance of the strain, bioflocculant production and flocculation assay which were common in all experiments.

Chapter 4 covers the topic on factors affecting bioflocculant production by the strain in submerged fermentation which generally discussed on the fermentation conditions.

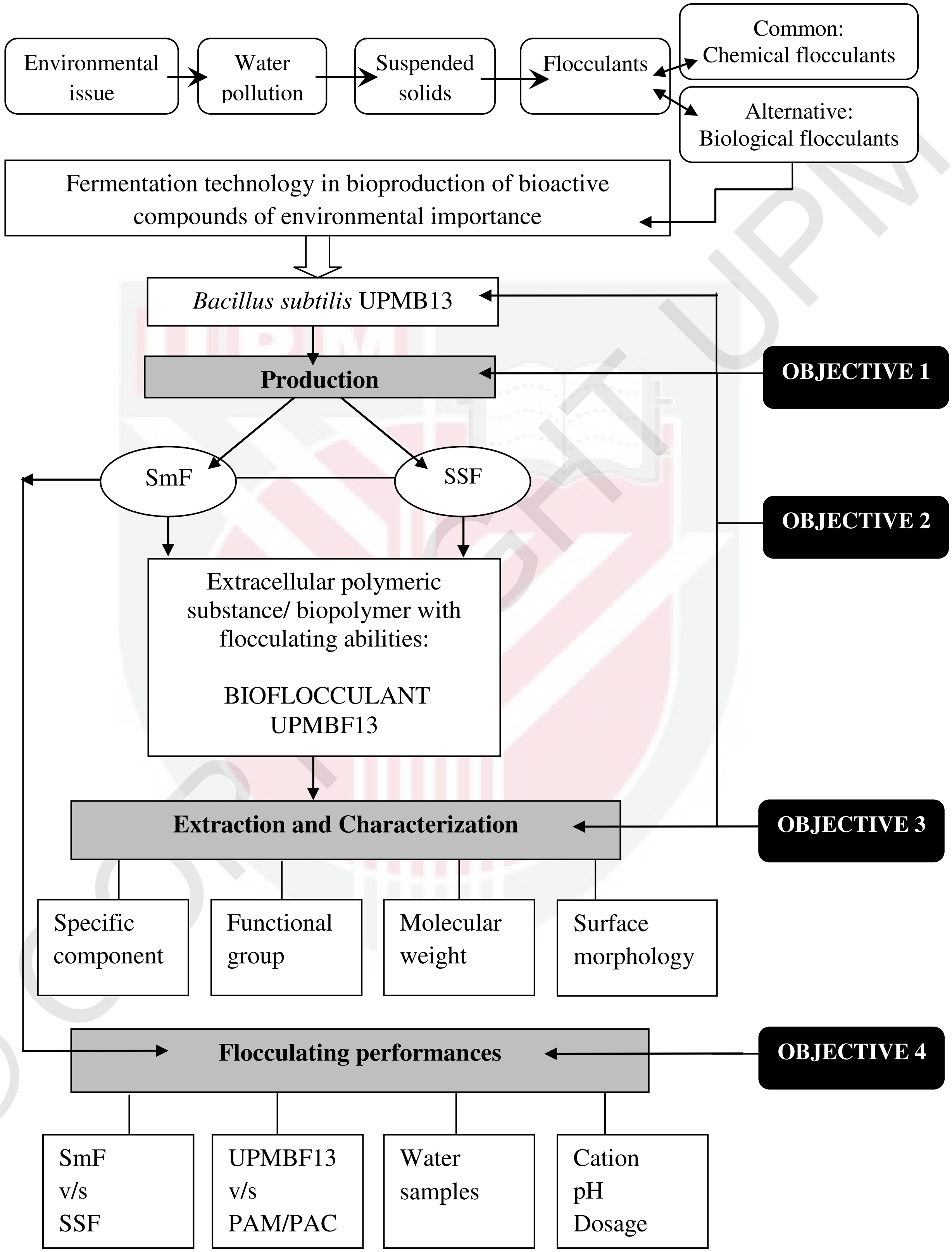
Chapter 5 describes the production of bioflocculant by *Bacillus subtilis* UPMB13 through solid-state fermentations in laboratory and pilot scale levels.

Chapter 6 portrays the extraction process and characterization of the extracted bioflocculant which constitute the chemical and also morphological characteristics of the bioflocculants from both fermentation methods.

Chapter 7 focuses mainly on the performances of the bioflocculants extracted from the submerged fermentation. The chapter will discuss the factors affecting the bioflocculants performance in kaolin assays and comparison of their capabilities with common commercial flocculants in a jar-test setup. Additionally, the chapter will also discuss the comparison of the performances of the extracted bioflocculants from both fermentation methods.

Chapter 8 summaries the research and discusses the overall findings, evaluation and the contribution of this research. Future prospect from the outcome of this research are conversed as well.

1.7 Conceptual Framework



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