BIOFLOCCULANT PRODUCTION BY *Aspergillus flavus* USING CHICKEN VISCERA AS A SUBSTRATE

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ABSTRACT

The biodegradability and safety of the bioflocculants make them potential alternative to non-biodegradable chemical flocculants for wastewater treatment. However, low yield and production cost has been reported to be the limiting factor for large scale bioflocculant production. The sustainability and economics of bioflocculant production is dependent on the use of low-cost substrate at optimum culture conditions. This study focused on the optimization of culture conditions for *Aspergillus flavus* growth and its bioflocculant production using chicken viscera hydrolysate as substrate. The effects of culture conditions including pH, shaker speed, temperature and inoculum size on bioflocculant production were investigated and optimized via response surface method in accordance with the critical component design (CCD) package of design expert. The purified bioflocculant was characterized using physical and chemical analysis. The flocculation performance and effect of cations on the bioflocculant was investigated using jar testing and Kaolin clay suspension as wastewater model. Under optimized culture conditions, 6.75 g/L of crude bioflocculant was produced. The bioflocculation activity was mostly distributed in the cell free supernatant with optimum efficiency of 91.8% at dose of 4 mL/100 mL Kaolin suspension. The purified bioflocculant was an uneven, coarse EPS assemblage in netted texture consisting of 23.46% protein and 74.5% polysaccharide, including 46% neutral sugar and 2.01% uronic acid with zeta potential of -25.28 ± 2.7 mV at pH 6.2. The Fourier-transform infrared (FTIR) and X-ray photoelectron spectroscopy (XPS) analysis indicated the presence of carbonyl, amino, hydroxyl and amide functional groups and mass proportion of C, O and N at 63.46%, 27.87% and 8.86%, respectively. It had a minimum of 83.1% efficiency in flocculating 2-12 g/L Kaolin clay suspension over a wide temperature range (4 - 80°C) and function optimally at neutral pH. It effectively flocculated different suspended particles such as activated carbon (92%), soil solids (94.8%) and algae (69.4%) at varying concentrations. Addition of both Ca^{2+} and Mg^{2+} stimulated the efficiency of the bioflocculant at all the concentrations tested with optimum flocculation efficiency of 95% recorded with 5 mL 1% Ca^{2+}. Lower concentrations (1-2 mL) of Al^{3+} also stimulated the bioflocculant to about 94%, K^{+} slightly enhanced the flocculation at 4 – 10 mL 1%, while Na^{+} and Fe^{3+} inhibited the flocculation. This study indicates high potential of cation dependent bioflocculant production from chicken viscera at appropriate culture conditions and stand as an attractive candidate for additional exploration and development for large-scale bioflocculant production and application.

ABSTRAK

Keterbiodegradan serta selamat digunakan telah menjadikan bioflokulan sebagai potensi alternatif kepada flokulan kimia yang tidak terbiodegrad bagi rawatan air kumbahan. Walau bagaimanapun, pengeluaran yang rendah serta kos penghasilan yang tinggi telah dilaporkan menjadi faktor penghad kepada pengeluaran bioflokulan pada skala besar. Kelestarian dan ekonomi pengeluaran bioflokulan bergantung kepada penggunaan substrat yang murah pada kadar kultur yang optimum. Kajian ini memfokus kepada pengoptimuman keadaan kultur bagi pertumbuhan *Aspergillus flavus* serta penghasilan bioflokulan menggunakan hidrolisat visera ayam sebagai subsrat. Kesan keadaan kultur termasuk pH, kelajuan penggoncang, suhu dan saiz inokulum pada pengeluaran bioflokulan telah disiasat dan dioptimumkan menggunakan kaedah permukaan respon selaras dengan pakej pakar reka bentuk komponen kritikal (CCD). Bioflokulan yang tulen dicirikan dengan menggunakan analisis fizikal dan kimia. Prestasi flokulasi dan kesan kation pada bioflokulan dikaji menggunakan ujian jar dan tanah liat Kaolin sebagai model sisa buangan. Di bawah keadaan kultur yang optimum, 6.75 g/L bioflokulan mentah dihasilkan. Aktiviti bioflokulasi kebanyakannya dihasilkan di dalam supernatan sel bebas dengan kecekapan optimum 91.8% pada dos 4 mL/100 mL larutan Kaolin. Bioflokulan yang ditulenkan adalah tidak sekata dengan pengumpulan EPS yang kasar di dalam tekstur jejaring terdiri daripada 23.46% protein dan 74.5% polisakarida, termasuk 46% gula semulajadi dan 2.01% asid uronik dengan keupayaan zeta -25.28 ± 2.7 mV pada pH 6.2. Inframerah empatier-transform (FTIR) dan spektroskopi fotoelektron X-ray (XPS) bagi bioflokulan tulen menunjukkan kehadiran kumpulan fungsi karbonil, amino, hidroksil dan amida dan nisbah jisim bagi C, O dan N masing-masing pada 63.46%, 27.87% dan 8.86%. Ia mempunyai kecekapan minimum 83.1% di dalam memflokulasi 2-12 g/L larutan Kaolin di dalam julat suhu yang luas (4-80°C) dan berfungsi secara optimum pada pH neutral. Ianya berkesan memflokulasi pelbagai partikel terlarut seperti karbon teraktif (92%), pepejal tanah (94.8%) dan alga (69.4%) pada kepekatan yang berlainan. Penambahan Ca^{2+} dan Mg^{2+} merangsang kecekapan bioflokulan di semua kepekatan yang diuji dengan kecekapan optimum flokulan 95% direkodkan dengan 5 mL 1% Ca^{2+}. Pada kepekatan rendah (1-2 mL) Al^{3+} juga merangsang bioflokulan kepada kira-kira 94%, K^{+} hanya meningkatkan sedikit flokulasi pada 4 – 10 mL 1%, manakala Na^{+} dan Fe^{3+} menghalang flokulasi. Kajian ini menunjukkan keupayaan tinggi bioflokulan berkation dari visera ayam pada keadaan kultur yang sesuai serta menjadi sumber penerokaan baru untuk pembangunan produksi dan aplikasi bioflokulan yang berskala besar.

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LIST OF ABBREVIATIONS

EPS	-	Extracellular Polymeric Substances
RSM	-	Response Surface Methodology
DLVO	-	Derjaguin, Landau, Verwey, and Overbeek
TSS	-	Total Suspended Solid
COD	-	Chemical Oxygen Demand
PAC	-	Polyaluminum Chloride
EDTA	-	Ethylene Diamine Tetraacetic Acid
SEM	-	Scanning Electron Microscopy
FTIR	-	Fourier Transform Infrared Spectroscopy
NMR	-	Nuclear Magnetic Resonance
ESEM	-	Environmental Scanning Electron Microscopy
CLSM	-	Confocal Laser Scanning Microscopy
QCM	-	Quartz Crystal Microbalance
GC	-	Gas Chromatography
HPLC	-	High-Performance Liquid Chromatography
GC-MS	-	Gas Chromatography-Mass Spectrometry
IHF	-	Incorporation Host Factor
POME	-	Palm Oil Mill Effluent
DCB	-	Divalent Cation Bridging
AOAC	-	Association of Official Analytical Chemists
UNiCC	-	Microbial Culture Collection Unit
PDA	-	Potato Dextrose Agar
CCD	-	Central Composite Design
OFAT	-	One Factor at a Time
ANOVA	-	Analysis of Variance
ESI	-	Electrospray Ionization
DLS	-	Dynamic Light Scattering

LIST OF SYMBOLS

KBr	-	Potassium Bromide
μ	-	Specific growth rate
X	-	Biomass
t_d	-	Doubling time
Y	-	Yield coefficient
S	-	Substrate

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Environmental issues and their related devastating influence on economics of most nations are universal challenges that requires much attention. Though water is an inevitable need for life and energy, millions of human races globally are still anguish with the scarcity of quality water for drinking and other purposes (Barbera and Gurnari, 2018). In fact, one of the greatest challenges facing the globe today is mainly pollution of water which has become a serious hindrance to quality of life most especially in the industrious communities where the accumulated wastes or pollutants can directly or indirectly find their way to the water bodies. The continuous unplanned urbanization and rapid population growth has led to increase water pollution and associated prevailing health challenges. Untreated harmful industrial discharges, agricultural and domestic waste constitute major source of water pollution. Pollution of water bodies can be subtly harmful to aquatic environment and pose challenge in attaining one of the world's longest goals of attaining portable water for domestic consumption.

In order to address the challenges of water pollution, a good number of treatment machineries including flocculation, ion exchange, filtration, oxidation processes and adsorption have been proposed and implemented. Flocculation involves removal of colloidal particles, cells and other suspended solids from a suspension in form of flocs due to aggregation. It has found wide application as a substitute to centrifugation and filtration in separation of microorganisms from broths beverages and pharmaceutical preparations (Okaiyeto et al., 2016). Flocculation is also an effective method in wastewater treatment for suspended solids and metal ion removal (Wu and Ye 2007). Over the years the chemical flocculants have widely been used to achieve flocculation due to their efficiency and cost effectiveness nevertheless, they are toxic to human health due to

the resultant secondary pollution created from residues of chemical flocculants (Bukhari *et al*., 2018).

Further, the polymeric substances produced by microorganisms as metabolites of their cellular growth are comprise of complex multi-chain, weighty molecular polymers with repeating units of branched sugar derivatives, glycoprotein, polyolses and proteins (Sheng *et al.*, 2010). Some of these polymers have ability to flocculate out suspended colloidal particles from wastewater and are generally refer to as bioflocculants.

Bioflocculant yield as a product of fermentation is no doubt affected by the culture conditions and substrate composition. The effect of culture conditions varies with the organism and the substrate composition. Though biodegradable to no harmful intermediates and hence produce no secondary pollutant (Sun *et al.*, 2015a), microbial bioflocculant production is associated with low yield, high cost of production and sometimes low efficiency. To make bioflocculant as alternative to toxic chemical flocculants and for large scale industrial application, research efforts are directed towards maximizing yield and reducing the production cost. These can be achieved through isolation of new bioflocculant producing microbes, search for low cost but nutrient rich substrates for fermentation and modification of culture conditions and growth parameters for new and existing substrates.

The present study uses chicken viscera to produce bioflocculant. The selection of the viscera is based on its composition of fermentable nutrients and availability in this part of the globe at no cost. Moreover, processing the viscera to fermentable nutrient for bioproduct production is simple, hydrolysis can be achieved at acidic pH with the indigenous enzymes and therefore no cost of enzyme is involve. Using the viscera for this purpose also offer a cost-effective, and eco-friendly alternative to manage the poultry waste. The bioflocculants produced with the use of waste generally have lower cost of production than the chemical flocculants that are produced via expensive techniques and raw materials. The cost of production is further reduced with the used of microorganisms which readily ferment the waste to bioflocculant without any prior pretreatment.

Design expert; a blend of mathematical and statistical methods, employed to evaluate the impacts of several independent variables on system response without any necessity to predetermined link between the objective role and variables. This method surmount the weaknesses of classical methods, and has been attested to be operational for optimization of target microbial metabolite yield (Ren *et al.*, 2013). This study applied critical component design and response surface methodology, RSM to optimize the pre-studied culture conditions for *Aspergillus flavus* growth and bioflocculant yield using chicken viscera hydrolysate as a sole media. The physical and chemical properties of the bioflocculant were also determined along with the flocculation behaviour of the bioflocculant.

1.2 Problem Statement

Industrial growth is receiving a continuous and desirable attention because of its role in economics of every nation. However, it has substantially contributed high amount of water pollutants in addition to domestic and agricultural wastes that are already of major concern. The pollution of water bodies is not only damaging to the aquatic organisms but also renders the water unfit for local usage. Utilization of polluted water results to waterborne infections commonly found in under develop and developing nations. Thus, there is needs for continuous effort for water treatment strategies and evaluation of existing strategies.

High efficiency and low cost of chemical flocculants have make them suitable for use in wastewater and drinking water treatment and other industrial processes that requires flocculation (Salehizadeh and Shojaosadati, 2001). However, their wide application has resulted in environmental and health impacts. For example, many reports and one of them by Adlard and Bush (2018) and Campbell (2002) have demonstrated association of aluminium salts with Alzheimer's disease. The use of aluminium as coagulating agent in water treatment can accumulate more aluminium in the treated effluent than in raw water. Similarly, neurotoxicity and carcinogenic effect of monomeric nonbiodegradable leftovers from polyacrylamide; an efficient chemical flocculant (LoPachin, 2004; Semla *et al.*, 2017) constitutes serious

disadvantage to their use for wastewater treatment. These foreseeable weaknesses related to chemical flocculants necessitate exploration for safe substitute (Nwodo and Okoh, 2013).

Although biodegradable, harmless and does not generate secondary pollutant, bioflocculants are associated with expensive cost of production and low yield. These limits their industrial applications. Therefore, there is need to search for bioflocculant yielding potential low-cost substrates as well as optimization of culture conditions for microbial growth on such substrates and corresponding bioflocculant yield. As different microorganisms and substrates are continuously been investigated to reduce production cost and increase the efficiency of bioflocculants as a promising substitute, nutrient sources, has been identified as one of major factors altering the economics of bioflocculant production especially for large-scale uses. Therefore, the feasibility of mass bioflocculant production is essentially coupled with the use of cheap nutrient sources such as those from animal waste. Recycling of agricultural waste has received much attention however, some of them such as chicken viscera are still underutilized. Although reported as a rich source of nutrient for microbial growth (Jamdar and Harikumar, 2008) there has not been any study to date that utilizes chicken viscera as a substrate for bioflocculant production. The chicken viscera in this study was hydrolyzed to make the nutrient readily available for microbial fermentation and to facilitate the measurement of the *A. flavus* growth. *A. flavus* was selected because it has been reported in the previous study (Aljuboori *et al*., 2015 and Aljuboori *et al.,* 2013) to produce bioflocculant using commercial media at specific culture conditions

Further, bioflocculants have been reported to be less efficient as compared to chemical flocculants. This is largely due to repulsive forces that exist between the negatively charged bioflocculants and the suspended particles in wastewater that are in most cases negatively charge too. Evaluation of the bioflocculant generated from chicken viscera in the presence of positively charge ions is needed to enhance flocculation efficiency. This is because the hybridized cations can reduce repulsion between the negatively charged bioflocculants and negatively charged suspended particles through bridging and neutralization.

1.3 Objectives

1. To hydrolyse and analyse chicken viscera for fermentable nutrient composition
2. To optimize culture conditions for *Aspergillus flavus* growth and bioflocculant production on chicken viscera hydrolysate via batch fermentation.
3. To characterize bioflocculant produced from chicken viscera hydrolysate
4. To evaluate the flocculation behaviour of the bioflocculant and effect of cationization on flocculation efficiency

1.4 Scope of the Study

The scope of this study covers hydrolysis of chicken viscera using the indigenous enzymes at acidic pH of 2.8. The optimization of culture conditions for *A. flavus* fermentation of chicken viscera hydrolysate for bioflocculant production was conducted using one factor at a time (OFAT) in which one parameter was varied while keeping the remaining constant. The parameters studied included incubation period, initial pH, shaker speed, temperature and inoculum size. Response Surface Methodology, RSM (mainly critical component design; CCD) was further applied in optimization process to produce more accurate, reliable and significant study. The parameters fed to the CCD were based on the values obtained from the OFAT study. The purified bioflocculant was characterized for surface morphology (scanning electron microscopy); functional groups (FTIR and XPS), composition analysis and flocculation assay all using standard techniques. The efficiency of the bioflocculant was stimulated via cationization with metal ions.

1.5 Significances and Original Contributions of This Study

This research will address the ongoing interest of producing a value-added bioactive compound (bioflocculant) from poultry waste as substrate that may

substitute the dependence on expensive commercial media that has add to cost of production of bioflocculant. In specific, this work brings into view the innovation of using chicken viscera for bioflocculant production for application in water treatment thereby offering a basic viewpoint for possibility of this innovation for imminent prospect of large scale bioflocculant production from chicken viscera and possibly for commercialization. Overall, this study will be beneficial to water treatment industry and contributes to the existing knowledge on valorisation of waste for bioflocculant production. A simple, inexpensive fermentation conditions for producing bioflocculant in aerated and agitated culture system was developed. The success in establishing substitution of commercial nutrient sources with chicken viscera for bioflocculant production offer a promising scientific wastewater treatment alternative that is cost-effective and eco-friendly while also combating environmental pollution from chicken viscera.

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LIST OF PUBLICATIONS

Journal with Impact Factor

1. **Mohammed J.N and Dagang W.R.Z.W** (2019). Culture Optimization for Production and Characterization of Bioflocculant by Aspergillus flavus grown on Chicken Viscera Hydrolysate. World Journal of Microbiology and Biotechnology DOI: 10.1007/s11274-019-2696-8 **(Q2, IF: 2.652)**
2. **Mohammed J.N and Dagang W.R.Z.W (2019).** Implications for Industrial Application of Bioflocculant Demands Alternatives to Conventional Media: Waste as a Substitute. Submitted to Water science and technology **(Q3, IF:1.624)**

Indexed Journal

1. **Mohammed, J. N.** and Dagang, W. R. Z. W (2019). Development of a new culture medium for bioflocculant production using chicken viscera. *MethodsX*. 6, 1467-1472. https://doi.org/10.1016/j.mex.2019.06.002 (**Indexed by SCOPUS**)
2. **Mohammed, J. N** and Dagang W. R. Z. W (2019): "Role of Cationization in Bioflocculant Efficiency: a Review." *Environmental Processes* 1-22. https://doi.org/10.1007/s40710-019-00372-z (**Indexed by SCOPUS**)

Non-indexed Journal

1. **Mohammed, J. N**. and Dagang, W.R.Z.W (2017). Fabrication of a Continuous Flow System for Biofilm Studies. *Nigerian Journal of Microbiology*, 31(2), 3933-3941.
2. **Mohammed, J.N**. and Dagang, W.R.Z.W (2017). Effect of surface roughness on susceptibility of Escherichia coli biofilm to benzalkonium chloride. *Malaysian Journal of Fundamental and Applied Sciences*, 13(1), 14-18.
3. Chicken Viscera hydrolysate as a sole source of media for bioflocculant production **(copyright). Nombor Pemfailan LY2018006152**

Indexed Conference Proceedings

1. **Mohammed, J. N.** and Dagang, W.R.Z.W (2019). Flocculation behaviour of bioflocculant produced from chicken viscera. In *E3S Web of Conferences* (Vol. 90, p. 01013). EDP Sciences. https://doi.org/10.1051/e3sconf/20199001013 (**Indexed by SCOPUS)**
2. **Mohammed, J. N.** and Dagang, W.R.Z.W. Effect of Cation on Efficiency of *Aspergillus flavus* Bioflocculant Produced from Chicken Viscera Hydrolysate. Presented in 3rd ASIA International Multidisciplinary Conference 2019 and submitted for publication in **Scopus indexed Journal**