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# Okara: a Promising Resource for Novel Bioflocculant Synthesis

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The Sustainable Development Goal no 12 (SDG 12) focuses on responsible consumption and production, which includes reducing waste generation, achieving environmentally chemical management, and encouraging companies to adopt sustainable practices. Okara, an insoluble residue from soybean processing, has been identified as a potential carbon and nitrogen resource for bioflocculants synthesis. This resource can reduce production costs which can make microbial bioflocculants more competitive, and reduce the amount of organic waste that ends up in landfills, where it contributes to the release of methane – a potent greenhouse gas. Bioflocculants can improve water quality by removing pollutants and contaminants from industrial wastewater. However, the low efficiency of bioconversion of okara to bioflocculants remains a major issue. This study focuses on investigating the potential of novel bioflocculant production of *Bacillus licheniformis* CGMCC 2876 using okara as an alternative carbon source and to optimize conditions for its use. The result showed that the use of okara as an alternative substrate resulted in 89.2 % flocculation activity of the kaolin suspension when $Ca^{2+}$ was used as a coagulant and pH was 12. By utilizing okara as a carbon source in novel bioflocculant synthesis, enhances waste management efficiency and reduces environmental impact, contributing to sustainable infrastructure development and cleaner manufacturing technologies that reduce pollution and industrial operations' carbon footprint.

## 1. Introduction

Bioflocculants are biopolymers predominantly synthesized by microorganisms or plants, that can aggregate or flocculate suspended particles in the liquid media, allowing for their easy removal and separation from the aqueous phase (Selepe and Maliehe, 2024). Bioflocculant can be effective in recycling suspended solids that are difficult to treat with conventional methods. The utilization of renewable resources to produce bioflocculants has gained significant attention due to the growing concern over environmental sustainability and the need to reduce greenhouse gas emissions (He et al., 2022).

Researchers have used wastewater from breweries, dairies, or fish meals as the medium for producing bioflocculants. Zhang et al. (2007) observed that brewery wastewater may support the growth of microorganisms and exhibit up to 96.8 % flocculating activity. Dairy wastewater may be used by *Klebsiella mobilis* to make bioflocculant with 95.4 % flocculating activity produced per L of broth (Wang et al., 2007). Agricultural wastes like rice stover and maize stover have also been used to create microbial bioflocculants as low-cost carbon

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sources. To lower the cost of manufacturing, a variety of wastewaters, including those from chromotropic acid and potato starch, have been employed as inexpensive carbon sources (Wang et al., 2013).
Okara is a byproduct generated during the production of soy-based foods such as tofu and soy milk. In countries like China, Japan, Singapore, significant quantities of okara are produced annually as result of these processes, with approximately 2.8 Mt in China, 0.8 Mt in Japan, 0.01 Mt in Singapore and 14 Mt in other parts of the world. 1 Mt of soybeans yields approximately 7 Mt of soymilk and 2 Mt of okara. Tofu manufacturing generates significant water waste, leading to okara and liquid waste. Okara compromises roughly 50 wt% carbohydrates (hemicellulose, cellulose, and lignin), 20-30 wt% proteins, and 10-20 wt% lipids. Okara, a cost-effective and sustainable substrate, provides carbon and nitrogen for microbial growth and bioflocculant production. Fresh okara contains a high moisture content (about 77.7 %) (Vong and Liu, 2016), present new challenges and limitations compared to traditional substrates. It can cause fast microbial deterioration and require urgent drying or preservation, leading to variable substrate quality (Bhagchandanii et al., 2020). To address these limitations, researchers have explored various strategies to optimize okara-based fermentation for improved bioflocculant yield and quality. Some studies have focused on supplementing the okara substrate with additional nutrients, such as carbon and nitrogen sources, to boost microbial bioflocculant synthesis, while others explore pretreatment methods to enhance nutrient availability (Naraian et al., 2016).
Okara fermentation requires effective pretreatment procedures like acid and enzymatic hydrolysis to enhance nutrient availability to microorganisms. The primary focus of dilute acid pretreatment is breaking down hemicellulose through hydrolysis, specifically to remove xylan from the hemicellulose portion. Dilute acid pretreatment at high temperatures is favored for increased cellulose hydrolysis (Johannes and Xuan, 2024). Enzyme hydrolysis, while longer and more expensive due to enzyme costs, is eco-friendly, produces higher product purity, and improves microbial efficiency due to less inhibitors (Woo et al., 2023).
*B. licheniformis* CGMCC 2876 is a Gram-positive bacterium with potential for producing bioflocculants due to its high flocculating activity, adaptability, and non-pathogenic nature. The strain can also be compatible with okara as an alternative carbon source, reducing waste, and lowering production costs, making it cost-effective for industrial applications (Xiong et al., 2010).
Limited research has been done on optimizing pretreatment procedures for bioflocculant synthesis using okara as a substrate. Due to the unique composition of okara, typical microbial strains may not work well. This study aims to explore the potential of okara as a sustainable resource for bioflocculant production and to optimize its use.

## 2. Methodology

### 2.1 Okara hydrolysate preparation

Okara was obtained from Fraser and Neave Holding Bhd, based in Shah Alam, Selangor, Malaysia. Okara biomass was washed and heated in an oven at 50 $^{o}$C for 24 h to eliminate the impurities and moistures, then crushed and sieved at 200 mesh. To ensure comparability, this study employed three pretreatment processes to pretreat the biomass. Acid pretreatment, enzyme hydrolysis, and acid-based pretreatment followed by enzyme hydrolysis are among the pretreatment procedures used. Dilute sulfuric acid was used on acid pretreatment, and enzyme hydrolysis using commercial cellulase Cellic® Ctec3 by Novozyme.

#### 2.1.1 Acid pretreatment
The crushed okara was hydrolyzed in dilute sulfuric acid (0.4 M) with a solid-liquid ratio of 1:10 at 121 $^{o}$C for 60 min. The mixture's pH was adjusted with 2 M NaOH until it reached 4.8 after acid hydrolysis. The supernatant was separated from the solid using a vacuum filtering apparatus, and the mixture was washed with 150 mL of distilled water. The dried solid was then placed in a desiccator for enzymatic hydrolysis, and the reducing sugar in the supernatant was determined using the dinitro salicylic acid (DNS) technique.

#### 2.1.2 Enzymatic hydrolysis of okara
Enzyme hydrolysis was carried out in 100 mL conical flasks. Before enzymatic hydrolysis, the biomass was rinsed three times with sodium citrate buffer (pH 4.8, 0.05 M). Enzymatic hydrolysis was performed for 72 h at 50 $^{o}$C with a shaking speed of 150 rpm and a solid loading of 10 %. Novozyme's Cellic® Ctec3 was employed in the experiment, with an enzyme loading of 20 FPU/g. After the enzymatic hydrolysis, sample were deactivated by placing them in water bath at 100 $^{o}$C for 10 min. Centrifugation at 12,000 rpm for 10 min separated the solid residue from supernatant. The total reducing sugar was calculated using the dinitro salicylic acid (DNS) technique, which used glucose as a reference. The solutions were then examined at 540 nm with a spectrophotometer (Mapada-V-1100D, Shanghai, China).

### 2.2 Experimental strain and culture media

The strain utilized in this study, known as *Bacillus licheniformis* CGMCC 2876, was came from our collaborator laboratory at Xiamen University's College of Chemistry and Chemical Engineering in China. B. *licheniformis* CGMCC 2876, was cultivated on Luria Bertani agar in a sterile petri dish and incubated at 37 °C for 24 h. The strain was then added to 50 mL of seed media in a 250 mL conical flask and cultured on a reciprocal shaker at 37 °C and 200 rpm. Following 36 h of incubation, the seed culture was used for further experiments. The components in the seed media were as follows (g/L): glucose (10), urea (0.5), yeast extract (0.5), $K_2HPO_4$ (0.1), $KH_2PO_4$ (0.1), NaCl (0.1), and $MgSO_4.7H_2O$ (0.2). For the fermentation process, the seed culture was inoculated at a volume of 4 % and agitated at a speed of 200 rpm for a total of 56 h in a 250 mL conical flask with 50 mL of fermentation media. The entire process took place at a temperature of 37 °C, ultimately resulting in the production of bioflocculant. The components in the fermentation media are as follows (g/L): glucose (13.9), urea (2.67), yeast extract (1.8), $K_2HPO_4$ (1.4), $KH_2PO_4$ (5.6), NaCl (2), and $MgSO_4$ (0.048). All the media had their pH set to 7.2. Distilled water was used to prepare all media, which were then sterilized at 121 °C for 15 min (Liu et al., 2017). Batch fermentation was occurred under the identical conditions as pre-cultivation. Samples (5 mL) were collected every 3 h and examined for cell growth, and flocculating activity. To optimize bioflocculant production, experiments were conduct using various okara hydrolysate and glucose ratio.

### 2.3 Determination of bioflocculant activity

After fermenting for 56 h of fermentation, the culture broths were centrifuged for 15 min at 8000 rpm to remove the cells. The crude products were precipitated by introducing three volumes of ethanol to the supernatant. Next, the crude products were dissolved in distilled water and then subjected to lyophilization to yield purified products. Cell-free culture supernatants were utilized to assess the flocculating ability of *B. licheniformis* CGMCC 2876's bioflocculant. The determination of flocculating activity (FA) involved the use of kaolin clay suspension as an indicator due to its uniform size distribution, inertness, and specific surface properties (Nieto et al., 2022).
To begin, combine 20 mL of kaolin clay suspension (5 g/L) with 0.5 g/L $CaCl_2$ as a cation and 1 mL of the bioflocculant sample. The mixture was gently mixed and let to stand for 5 min at ambient temperature. After 5 min, the turbidity (OD550) of the supernatant was measured with an ultraviolet spectrophotometer (Mapada-V-1100D, Shanghai, China). A control experiment was carried out using the same approach but with equal amounts of distilled water. The flocculating activity was assessed by measuring the reduction in turbidity of the supernatant, as shown in Eq(1) below (Lei et al., 2015):

$$Flocculation\ activity\ (\%) = \frac{A-B}{A} \times 100\ \% \quad (1)$$

In the equation above, A and B represent the control and sample optical densities at 550 nm. Measurements were conducted in triplicate. To study the impact of the pH environment on bioflocculant activity, the pH of the suspension containing kaolin clay was adjusted by using 1 M solutions of HCl and NaOH. The pH levels tested ranged from 3 to 12, with increments of 1 pH unit.

## 3. Results and Discussion

### 3.1 Characteristics of biomass

Biomass characterization is essential in biochemistry, ecology, and environmental studies, analyzing chemical composition of biomass, especially carbon (C), hydrogen (H), nitrogen (N), and sulfur (S), to asses energy potential, nutritional composition, and environmental impacts. Table 1 shows the elemental composition of the okara used in this study.

*Table 1: Biomass characterization using CHNS analysis*

| Element | Okara (%) |
|---|---|
| C | 48.17 |
| H | 6.88 |
| N | 3.58 |
| S | $48 \times 10^{-3}$ |

### 3.2 Okara pretreatment

Okara, a soybean byproduct, is high in protein and dietary fiber, but its utilization is limited due to anti-nutritional factors and indigestibility. This study investigated various pretreatment methods, including dilute acid and enzymatic treatment using Cellic® Ctec3 from Novozyme. Dilute acid pretreatment involves treating okara with

a weak acid solution at an elevated temperatures (50 °C) to break down its complex structure, hydrolyzing hemicellulose into sugars and removes lignin, making the cellulose more accessible for enzymatic digestion, but can produce inhibitory compounds affecting microbial activity. This process can release up to 50-70 % of the total sugars from lignocellulosic biomass. Cellic® Ctec3 from Novozymes is a cocktail enzyme that contains cellulases, hemicellulases, and β-glucosidases. Cellic® Ctec3 is primarily used to hydrolyze lignocellulosic biomass and create fermentable sugars. The enzymes operate together to efficiently breakdown cellulose and hemicellulose in okara, resulting in a larger yield of fermentable sugars than single-enzyme (Luthfi et al., 2019). Cellic® Ctec3 is adaptable and effective on a wide range of lignocellulosic substrates, making it ideal for okara, which is made up of cellulose, hemicellulose, and residual lignin. It achieves up to 80-90 % sugar release from lignocellulosic biomass, with microbial activity enhanced by the absence of inhibitors. The combine approach of acid pretreatment followed by enzymatic hydrolysis leads to the highest bioflocculant production among the three methods as indicated in Table 2 below with sugar release efficiencies up to 90-95 %. The combined pretreatment method significantly increased sugar release efficiency by 42.6 % compared to dilute acid pretreatment alone and 16.5 % compared to enzymatic hydrolysis alone. The synergistic effect of the combined approach is significant to enhance the overall hydrolysis process.

*Table 2: Sugar recovery from okara pretreatment*

| Pretreatment condition | Sugar release (g/L) |
|---|---|
| Ground okara (control) | 0.421 |
| Okara + $H_2SO_4$ | 7.373 |
| Okara + Ctec3 | 10.515 |
| Okara+ $H_2SO_4$+ Ctec3 | 12.252 |

**3.3 Effect of carbon source on bioflocculant production by Bacillus licheniformis CGMCC 2876**

The media composition and fermentation conditions can impact bacterial secondary metabolites formation. Glucose provides a consistent nutrient supply for microbial growth, high biomass production, and bioflocculant production. Okara's high carbohydrate and protein content support *B. licheniformis* CGMCC 2876 growth, leading to increased biomass production and bioflocculant yield. To evaluate the influence of carbon source on bioflocculant formation, okara was processed using a combination of acid and enzymatic hydrolysis. This method was chosen due to its high efficiency in sugar release compare to the other pretreatment methods. Combining glucose and okara hydrolysate provides a balanced nutrients and enhance microbial metabolism and stimulate higher bioflocculant production by promoting diverse metabolic pathways, leading to better structural properties and higher efficiency in binding and aggregating particles (Lou et al., 2021). This study evaluated various carbon source compositions and found that different compositions had an impact on bioflocculant production. Figure 1 displays the impact of different composition of carbon supplies on bioflocculant production.

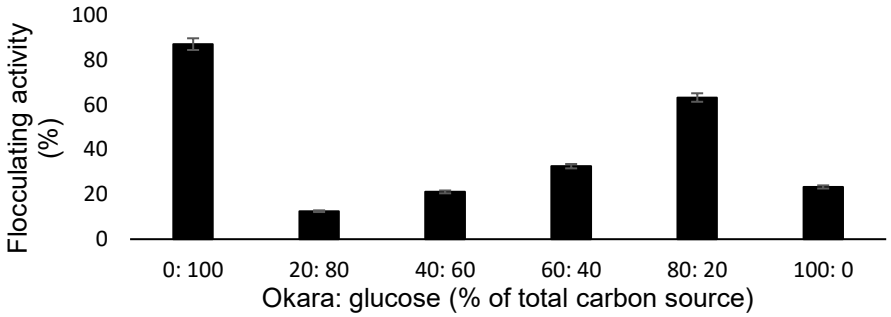


*Figure 1: Effect of different carbon source ratio on the bioflocculating activity*

In the beginning, glucose was shown to be the most effective carbon source for bioflocculant synthesis by *B. licheniformis* CGMCC 2876, with 86.9 % flocculating activity, followed by co-substrate 80 % okara hydrolysate and 20 % glucose, with 63.15 % flocculating activity.

Ctec3 enzyme is used in okara hydrolysis to convert lignocellulosic into fermentable sugar, including cellulose, hemicellulose, and pectin, yielding glucose, xylose, arabinose, galacturonic acid, mannose, and galactose.

In many bacteria, glucose is the preferred carbon source. This study found that the presence of xylose may prevent the bacteria from consuming glucose in mixed sugar fermentation due to various reasons, including metabolic control, transport efficiency, energy considerations, environmental adaptations, and specialized

regulatory systems (Domingues et al., 2021). In this study, the flocculating activity of the bioflocculant generated by *B. licheniformis* CGMCC 2876 in the presence of okara hydrolysate and glucose as carbon sources improved with increasing okara concentration, with 80 % okara content providing the optimal flocculating activity.

### 3.4 Effect of pH of kaolin suspension on bioflocculation activity

The pH of the environment significantly impacts the flocculating activity of a bioflocculant, as shown in Figure 2. The bridging mechanism for effective flocculation is affected by varying electrical charges at different pH levels.

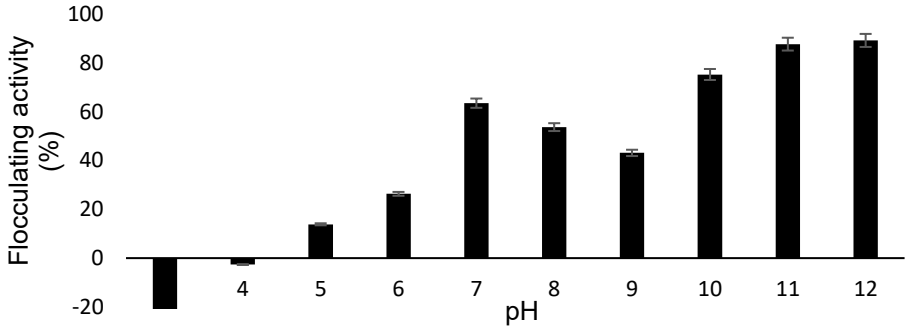


*Figure 2: Effect of pH of kaolin suspension on the bioflocculating activity when using 80 % okara hydrolysate and 20 % glucose as carbon source*

Bioflocculants respond differently to the electrical charge around suspended particles at different pH levels. The bioflocculant performed effectively in alkaline conditions, with a significant increase at pH 12 (89.19 ± 0.02 %) compared to other tested pH. Alkaline conditions, with a significant increase at pH 12, are more effective than acidic conditions. This is due to the presence of stable polysaccharides, proteins, and glycoproteins with ionized functional groups. Alkaline condition improves polymer chain extension for bridging mechanisms, increase solubility, availability of active sites, and allow bacteria to adapt to alkaline environment. Acidic instability occurs when these group become protonated, reducing their interaction with suspended particles. These variables all contribute to the higher effectiveness of bioflocculant activity in alkaline environment compared to neutral or acidic conditions (Salehizadeh and Shojaosadati, 2001). These findings aligns with research from Zheng et al. (2008), which found that alkaline pH supports bioflocculant synthesis by *Bacillus* sp.; in contrast, acidic conditions entirely hindered bioflocculant production.

Okara-derived bioflocculants improve water treatment flocs structural integrity, offering sustainable solutions in water treatment, agriculture, and environmental remediation. Optimization strategis include controlled heating, enhancing mechanical strength through cross-linking agents, functionalizing surfaces, natural fibers incorporation (Abdullah et al., 2024), or blending with other biopolymers (Rosmmi et al., 2021). These eco-friendly solutions align with circular economy principles and promote sustainable industrial practices.

## 4. Conclusions

Okara, an agricultural waste product, can be used as a carbon source in bioflocculant fermentation, reducing environmental impact and reliance on synthetic carbon sources such as glucose or sucrose. Pretreatment of okara is crucial for its digestibility and availability. Combining acid pretreatment using dilute acid with enzymatic hydrolysis provides the highest sugar recovery from okara (12.2 g/L) among other pretreatment methods. The optimal okara hydrolysate concentration for glucose substitution is 80 % with a fermentation period of 56 h and pH of 7.2. The maximum flocculating activity is 89.19 % in a pH environment of 12. *B. licheniformis* CGMCC 2876 generated a novel bioflocculant with an optimal pH of 12, suitable in industries with high-pH effluent, enhancing efficiency, cost-effectiveness, and environmental sustainability.

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