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Physical Parameters Optimization of Bacterial Cellulose from *Komagataeibacter sucrofermentans*

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ABSTRACT

Many studies have been concerned with nanocellulose's potential to produce environmentally friendly nanomaterial fibers. Bacterial cellulose has shown superiority over plant cellulose, leading to increased research focus on bacterial cellulose production. Among bacterial species, *Acetobacter*, particularly *Komagataeibacter* (formerly *Gluconacetobacter*), has captured interest due to its enhanced bacterial cellulose (BC) production and strain stability. Optimizing production processes becomes imperative with the growing demand for BC in various industries. This study explores the optimization of physical conditions for BC production using *Komagataeibacter sucrofermentans*. Five parameters—pH, temperature, aeration rate, shaking rate, and surface area, were examined using the One-factor-at-a-time (OFAT) method. This method was selected as it is useful in early-stage optimization to understand the effect of individual factors on BC production. The extracted BC was purified with 4.0 M NaOH solution at 80°C, and wet and dry weights were measured. Analysis via ANOVA determined the significance of each parameter in enhancing BC yield. Optimized conditions from this experiment —pH 5, temperature 20°C, 60% aeration rate, slow agitation (50 rpm), and large surface area fermentation (63.62 cm^2) shown to give better BC production. These findings have substantial implications for enhancing BC production efficiency on an industrial scale.

Keywords: Acetobacter, bacterial cellulose, environmental, fiber, industrial, optimization, pollution, polymer

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INTRODUCTION

Nowadays, the extensive use of fossil-based chemicals in sectors such as textiles, packaging, medicine, cosmetics, and other contemporary applications has resulted in pollution (Samanta & Das, 2021; Clews, 2016). Thus, utilizing environmentally friendly, renewable, and sustainable materials has gained increasing significance in creating diverse high-value goods with minimal environmental impact (Gupta & Pathak, 2020). Consequently, the search for alternatives has drawn considerable interest from academics and industry stakeholders, as these materials offer a substitute for diminishing non-renewable resources, environmental degradation, global warming, and energy scarcity. In this regard, cellulose, starch, alginate, chitin, chitosan, and gelatine have emerged as promising candidates due to their abundant availability from various sources (Trache, 2018). Among these, cellulose is the most abundant renewable compound derived from the biosphere, present in plants, algae, and certain bacteria (Trache et al., 2020). Bacterial nanocellulose was introduced as a non-toxic material to substitute the hydrocarbon-based material used in many products. The demand for bacterial nanocellulose or bacterial cellulose is increasing in the global market as it can be used in various applications. The United Kingdom has become the largest consumer of nanocellulose, followed by North America. In the Asia Pacific, South Korea, India, and Malaysia have also become countries that have rapidly uptaken this environmentally friendly material (Grand View Research, 2021). Hence, demands for bacterial cellulose across various industries have increased remarkably over the years.

Cellulose is generally found in plants, the main constituent of plant cell walls. It is a polymer composed of D-glucopyranose units linked by β-1,4-glycosidic bonds. Due to intra and intermolecular hydrogen bonds from the hydroxyl group present in cellulose, it forms various crystalline arrangements (Martins et al., 2011; Park et al., 2010). However, certain types of bacteria also can produce cellulose that has the same polymer composition in plants but differs in its characteristics. Some bacteria have the ability to synthesize cellulose by absorbing glucose, which are usually gram-negative bacteria (Swingler et al., 2021). Bacteria such as *Rhizobium*, *Agrobacterium*, *Salmonella*, and *Alcaligenes* are known to exhibit cellulose-producing capabilities. Notably, *Acetobacter*, now referred to as *Komagataeibacter*, is recognized as a prominent cellulose producer (Barja, 2021; da Gama & Dourado, 2018). BC is devoid of lignin, hemicellulose, and pectin; hence, it is considered to have high purity, leading to a higher degree of polymerization and crystallization at 40.6% to 83.4% (Pham & Tran, 2023; Naomi et al., 2020; Moniri et al., 2017). The crystallinity of wood fibers ranges from 55 to 70%, and natural plant fibers range from 60% to 70%, which shows that the crystallinity of cellulose from plants is much lower than BC (Jakob et al., 2022; Petroudy, 2017). These characteristics make BC resistant to wet conditions and have high elasticity and conformability.

Due to its superior characteristics, bacterial cellulose (BC) has become a product of interest to many industrial sectors seeking sustainable materials on par with petrochemicals. Especially in biomedical industries that have an increasing demand for BC materials (Choi et al., 2022). Bacterial cellulose is highly biocompatible, biodegradable, and non-toxic, which makes it a very competent material for pharmaceutical and biomedical industries. Due to its high porosity and ability to prevent microbial infection, it has become interesting and has high potential for biomedical applications. An example of BC application in biomedical is wound dressing. For example, BC is heightened with an antibacterial function that inhibits and suppresses bacterial growth in wound dressing (Volova et al., 2018; Deshpande et al., 2023). Not only that, BC can also be used for drug delivery, regeneration of bone, and as a biosensor (Deshpande et al., 2023). In a review by Bianchet et al. (2020), interest in BC for cosmetic applications has significantly increased over the years. Biotechnology advancements have led to the development of BC sheets tailored for the cosmetics and pharmaceutical industries, including innovations like antioxidant nanocellulose and vitamin B-loaded formulations (Deshpande et al., 2023; Bianchet et al., 2020; Volova et al., 2018). However, BC's potential goes beyond these industries; it is additionally marketable for use in culinary applications, such as packaging. Its thin, porous, reticulated structure efficiently filters out dust, fungi, and microbes, extending the shelf life of food that has been stored. According to Poyrazoğlu et al. (2021), sausages wrapped in BC film have lower microbial loads than those wrapped in cling film or left uncovered, proving that BC film can extend the shelf life of food. Compared to cling wrap made from petrochemicals, the web-like structure of BC provides better filtration against airborne pollutants (Choi et al., 2022; Poyrazoğlu et al., 2021). Furthermore, BC can be used as an organic polymer in the paper and pulp business and as a biotechnological polymer in the fabric industry (Coseri, 2021; Lahiri et al., 2021). Therefore, this demonstrates that BC offers numerous advantages and significantly influences various industries, contributing to the development of economically viable and sustainable materials for the benefit of society.

Physical conditions or the environment of bacteria culture become one of the important factors in the production of BC. It has been known that environmental conditions usually affect the activity of bacteria. Physical conditions such as pH, temperature, aeration rate and agitation rate play some roles in the production of BC. The pH conditions observed in conducted studies revealed that a pH range of 4.5 to 6.0 could efficiently induce the production of BC by several *Acetobacter* species, such as *Acetobacter xylinum* BRC5 and *Acetobacter senegalensis* MA1 (Hasanin et al., 2023; Aswini et al., 2020; Hwang et al., 1999). *Acetobacter* sp. was able to produce acid by converting glucose to gluconic or acetic acid. This resulted in a change in the pH condition of the media, resulting in a decline in pH levels. Although BC production is hindered at the lowest pH level, acid production may yield greater benefits by lowering the initial higher pH level to conditions

favorable for BC production (Victor et al., 2018; Siew, 2012). Thus, maintaining certain pH conditions for the fermentation process is essential to keep the efficient production of BC on an industrial scale.

Temperatures are essential in promoting the growth of bacteria and cellular activities. In a study investigating cellulose production from bacteria isolated from rotten fruit, the highest cellulose yield was observed at a temperature of 30°C, demonstrated by *Glunconacetobacter* sp. RV28, *Pseudomonas* sp. RV14, and *Enterobacter* sp. RV 11. This shows that most bacteria of different species have an optimal temperature of producing cellulose at 30°C (Rangaswamy et al., 2015).

Aeration plays a crucial role in supplying oxygen with the efficient cellular activities of bacteria. Optimal BC production occurs within a medium range of aeration. High BC yields were observed within the range of 3 L/min to 6 L/min aeration, as Krusong et al. (2021) and Shavyrkina et al. (2021) reported. Under low oxygen levels, cellulose production is constrained due to depleted oxygen content, resulting in reduced cellulose production. Conversely, excess oxygen acts as a proton acceptor at higher concentrations, converting glucose to gluconic acid and diverting the production pathway away from cellulose production (Tantratian et al., 2005). Agitated culture is preferred in industrial production as it can be mass-produced, and the process is quick (Lahiri et al., 2021). However, it causes a low degree of polymerization and exhibits a lower crystallinity level than the cellulose produced in static fermentation (Lahiri et al., 2021; Watanabe et al., 2007). It also induces mutant cells into non-cellulose-producing cells, which die due to shear stress (Buldum et al., 2018; Campano et al., 2015). Static culture has a low possibility of mutant cells and better steady production of BC. Nonetheless, static culture has a slower process and limited oxygen supply as the production only occurs on the surface of the liquid area (Ul-Islam et al., 2015).

While many studies have focused on other bacterial cellulose producers, fewer investigations have aimed to optimize conditions for improving BC yield from *K. sucrofermentans*. *Komagataeibacter sucrofermentans* has gained significant interest because of its superior BC production capabilities compared to its predecessor, the *Acetobacter* species. Thus, the primary objective of this study is to evaluate the influence of various physical parameters such as pH, temperature, shaking rates, and aeration on BC production by *K. sucrofermentans*. Using the one-factor-at-a-time (OFAT) approach, the study aims to analyze the effects of these physical conditions on bacterial culturing for enhanced BC production. This approach was chosen because it is useful in early-stage optimization where the effects of all the said parameters need to be understood before moving on to more complex statistical methods like response surface methodology (Tajik et al., 2024; Bhaturiwala et al., 2022). Parameters such as surface area were added to observe how the size of the container may affect BC production.

MATERIALS AND METHODS

The commercialized bacteria strain, *K. sucrofermentans* JCM 9370, was purchased from The American Type Culture Collection (ATCC), Gaithersburg, Maryland. It was grown on a glucose-yeast extract-calcium carbonate (GYC) agar plate prepared by and obtained from the Institute of Bioscience (IBS) Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia.

Agar and Media Preparation

K. sucrofermentans grew up in Hestrin-Schramm media (HS). HS media contained peptone (2.5 g), yeast extract (2.5 g), disodium hydrogen phosphate (Na_2HPO_4) (1.35 g), citric acid (0.7 g), magnesium sulfate ($MgSO_4$) (0.6 g), in distilled water (dH_2O) (0.3 L)—additional agar (7.5 g) for agar plate. In preparing HS media, 10% of the glucose solution was prepared separately and autoclaved. All media prepared were autoclaved at 121℃ for 15 min. All media were stored in the chiller at 4℃.

Preparation of Inoculum

K. sucrofermentans stock was streaked on an HS agar plate and incubated at 30°C for 4 days. Then, a single colony was inoculated in HS broth and incubated at 30°C, shaking rate at 150 rpm for 3 days. The growth of bacteria was noted with the formation of cellulose in broth with no or less cloudy conditions. A 10% (v/v) inoculum of *K. sucrofermentans* was inoculated to the HS medium for each of the parameters experimented on.

Bacterial Cellulose Culture Using One-factor-at-a-time (OFAT)

Preparation of Different pH for BC Production

Four different pH levels were established for each prepared HS medium—pH 5, pH 6, pH 7, and pH 8 in each flask. Sodium hydroxide (NaOH) was employed to elevate the pH, while hydrochloric acid (HCl) was used to lower it. Each flask was incubated for seven (7) days at 30°C. After seven days, the production was observed.

Preparation of Different Temperatures for BC Production

Four flasks were prepared with HS medium, and the pH was standardized to 5. Inoculum of *K. sucrofermentans* was cultivated into each flask, which was labeled with different temperatures: 20°C, 25°C, 30°C, and 35°C. The flasks were incubated at the temperature labeled for 7 days. After 7 days, the production was harvested and observed.

Agitated Culture Fermentation

Three flasks with HS medium were prepared and labeled with 0 rpm, 50 rpm, 150 rpm and 250 rpm, with pH and temperature standardized to pH 5 and 30℃. They were left incubated for 7 days (Pa'e et al., 2007).

Preparation of Different Aeration Rates for BC Production

Four flasks were prepared with 20%, 40%, 60% and 80% of aeration and labeled according to the percentage of aeration. The aeration of percentage was calculated using Equation 1:

$$\text{Aeration (\%)} = \frac{\text{Volume of flask - Volume of culture broth}}{\text{Volume of flask}} \times 100\% \quad [1]$$

The pH and temperature were standardized to pH 5 and 30℃, respectively. It was left incubated for 7 days.

Preparation of Different Surface Areas for BC Production

Following the size of the fermentation flasks, different surface areas were prepared. The bottom flasks were measured to determine the area value of each flask, which were 19.63 cm^2 (50mL flask), 28.27 cm^2 (250mL flask), 44.18 cm^2 (500mL flask), and 63.62 cm^2 (1000mL flask). Each flask was prepared with 20% HS media with pH and temperature standardized to pH 5 and 30℃. It was left incubated for 7 days in static condition.

Purification of Bacterial Cellulose

The bacterial cellulose membrane was harvested from the flask and cleaned with distilled water to remove excess media. Then, it was soaked in 1M NaOH solution for 30 min at 80°C to kill the bacteria and completely remove the cells and medium embedded in it. The BC membrane was left to cool down at room temperature. Then, it was soaked for 3 days with deionized water to further clean the BC sheet from any remaining excess media and bacterial cells on the cellulose fiber (Kiziltas et al., 2015). After 3 days, the extracted BC was dried in an oven at 100℃ for 30 minutes.

Measuring of Wet Weight and Dry Weight Cellulose

Harvested BC were immediately weighed, and the reading was recorded as wet weight. After oven-drying, the dry-weight BC was recorded. The wet weight of BC indicates the capacity of water it can hold, resulting in swelling. Dry BC measures the weight of pure cellulose produced (Bodea et al., 2021; Pa'e et al., 2007).

Statistical Analysis

Comparison of BC yields obtained was subjected to One-way analysis of variance (ANOVA) using Microsoft Excel 365. A post-hoc test was done to compare the significance of the value between the means of each parameter (Nagmetova et al., 2020).

RESULTS AND DISCUSSION

The Production of Bacterial Cellulose in Different pH

Figure 1 illustrates BC's wet and dry weight profiles obtained from a seven-day fermentation process conducted in various pH mediums. Among the four levels of pH that were experimented on for the production of BC (pH 5, pH 6, pH 7 and pH 8), pH 5 demonstrated the highest yield in both wet and dry BC, followed by the remaining pH levels.

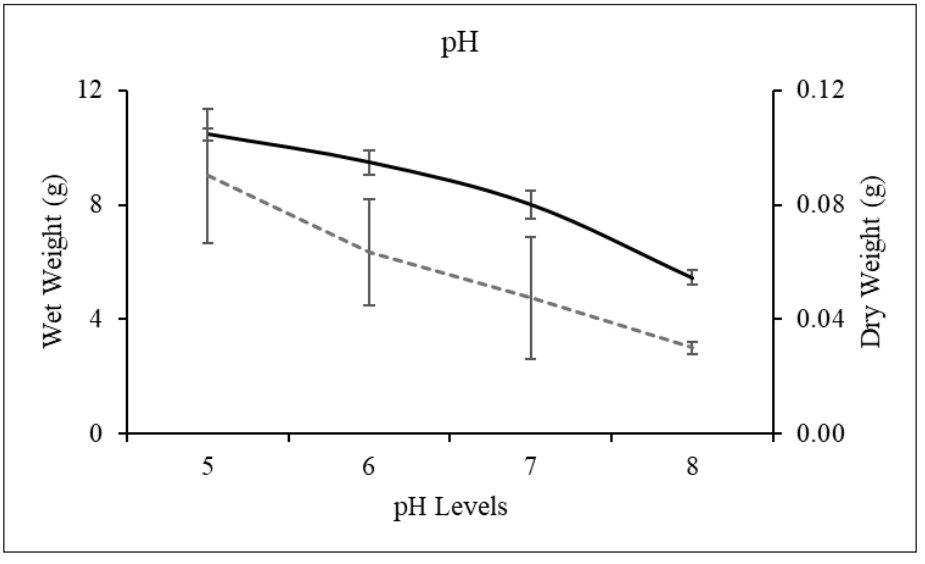


Figure 1. BC production by *K. sucrofermentans* at different pH conditions from pH 5 to 8. The graph shows the weight of BC; The smooth line denotes the wet weight of BC, and the dashed line denotes the dry weight of BC

Acetic acid bacteria efficiently produce cellulose at low pH levels ranging from 5 to 6.5 (Gomes et al., 2018). Referring to the data in Figure 1, *K. sucrofermentans* can efficiently produce BC at pH 5. The weight of BC obtained at pH 5 was approximately 10.5 g for wet BC and 0.09 g for dried BC. As pH increased, BC production was decreased but not entirely inhibited. At pH 8, BC was still produced but exhibited the lowest yield (within the pH range studied), with approximately 5.5 g for wet BC and 0.03 g for dried BC—half of the weight obtained at pH 5. This suggests that higher pH levels are not optimal for achieving high BC production. This was supported by ANOVA statistical analysis

revealing a calculated *p-value* of 0.0006, significantly below the critical threshold (*p-value* < 0.05), suggesting a significant difference in the influence of pH conditions on BC production. Nonetheless, by post-hoc Tukey test, a comparison of mean that has no significant difference is between pH 5 and pH 6 (*q-value = 0.98* < 1.48, T-value = 1.48) as well as pH 6 and pH 7 (*q-value = 1.47* < 1.48, T-value = 1.48) This can be seen in Table 1.

The impact of pH on BC production varies depending on whether the cellulose is in its wet or dry form. In wet BC, pH levels significantly influence the production process, with distinct variations observed across different pH values (Table 1). However, in the case of dry BC, post-hoc analysis indicates that pH does not have a significant effect on the formation of the dried membrane. This suggests that while pH plays a critical role during the initial synthesis and hydration stages of BC, its influence diminishes once the cellulose has dried.

Table 1
Post hoc Tukey test, comparison between the mean of each pH to the production of BC

Wet BC	**k**	**df**	**q**	**T**
	4	4	5.757	1.483
	pH	**$\lvert\bar{x}_1 - \bar{x}_2\rvert$**		***Significant diff.**
	5v6	0.976		q<1.483
	5v7	2.448		q>1.483
	5v8	5.001		q>1.483
	6v7	1.473		q<1.483
	6v8	4.026		q>1.483
	7v8	2.553		q>1.483
Dry BC	**k**	**df**	**q**	**T**
	4	4	5.757	0.0745
	pH	**$\lvert\bar{x}_1 - \bar{x}_2\rvert$**		***Significant diff.**
	5v6	0.0267		q<0.0745
	5v7	0.0426		q<0.0745
	5v8	0.0601		q<0.0745
	6v7	0.0159		q<0.0745
	6v8	0.0334		q<0.0745
	7v8	0.0175		q<0.0745

*q-value < T-value has no significant difference

A study by Hasanin et al. (2023) also achieved pH 5 to be an optimal condition for producing high BC yields. However, a study by Aswini et al. (2020) reported a high BC yield at pH 4.5. Moreover, after 7 days of fermentation, all culture pH levels were reduced to pH 3, indicating that *K. sucrofermentans* produced acid alongside BC production. It is critical that they utilize glucose and convert it into gluconic acid, thereby reducing pH in the media (Victor et al., 2018). Although this phenomenon contributes to BC production at high pH levels, synthesis of BC is inhibited as the pH reaches 3 (Siew, 2012). Further studies on lower pH values and variations in pH conditions may provide insight into BC production.

The Production of Bacterial Cellulose in Different Temperatures

The weights of wet and dry BC obtained from different temperatures are depicted in Figure 2. Four different temperatures condition was tested on the production of BC, which were 20℃, 25℃, 30℃ and 35℃. *Komagataeibacter spp.* can grow and produce BC at temperatures ranging from 28℃ to 30℃ (Cannazza et al., 2021; Marič et al., 2020).

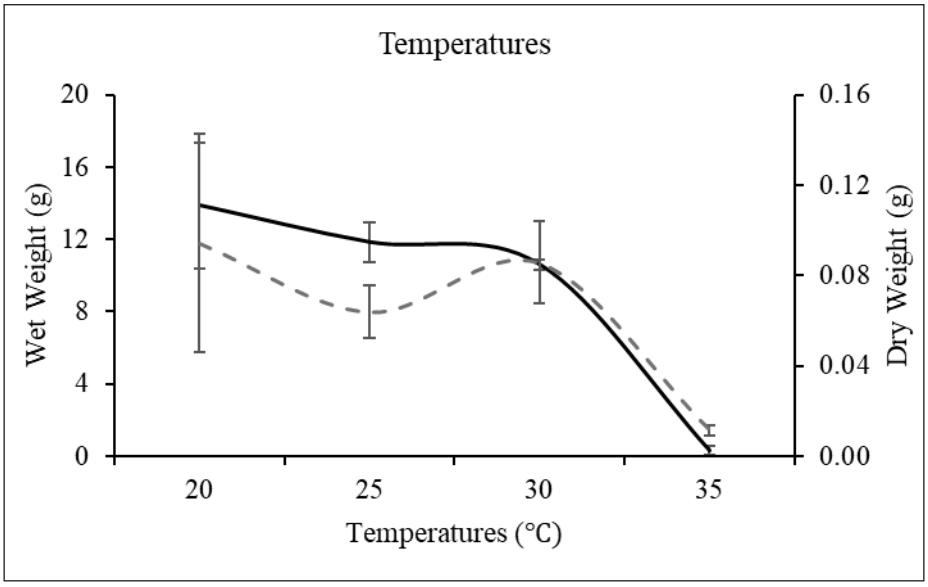


Figure 2. Production of BC by *K. sucrofermentans* in different temperature conditions from 20°C to 30°C. The graph shows the weight of BC; The smooth line denotes the wet weight of BC, and the dashed line denotes the dry weight of BC

The wet weight of BC from 20°C can be observed to be the highest compared to other temperatures (Figure 2). The total weight of wet BC obtained from 20°C culture fermentation was 13.9 g, while the dry BC weight obtained was 0.09 g. Both wet and dry weight BC obtained from 30°C was 10.6 g and 0.08 g, respectively. This finding contradicts previous reports on the influence of temperature conditions on BC production. Reshmy et al. (2021) indicated that the production of BC by *Acetobacter* sp. occurred at 30°C. In a study by Zakaria and Nazeri (2012), the maximum BC from *Acetobacter xylinum* was achieved at 30°C. Most of the conducted studies have also reported that the optimum condition of BC production ranges from 28°C to 30°C (Lahiri et al., 2021). However, a wide error bar was shown at 20°C (Figure 2). This indicates that the data achieved for BC produced at 20°C has less certainty (Cumming et al., 2007). The BC produced at 30°C was the second highest and had a smaller error bar which tells the data have more certainty and the results were less likely to happen by chance (Cumming et al., 2007). This may explain why *K. sucrofermentans* can produce efficiently and consistently at 30°C; however, this new finding can also signify that *K. sucrofermentans* may have different optimal temperature conditions in producing high-yield BC. Further study of BC production by *K. sucrofermentans* at lower temperatures should be done to gain better insight into the effect of temperature on BC production from this strain.

Table 2
Post hoc Tukey test, comparison between the mean of each temperature to the production of BC

Wet BC	k	df	**q**	**T**
	4	8	4.529	7.249
	Temperature (°C)	$\lvert\bar{x}_1 - \bar{x}_2\rvert$		***Significant diff.**
	20v25	2.028		q<7.249
	20v30	3.266		q<7.249
	20v35	13.560		q>7.249
	25v30	1.238		q<7.249
	25v35	11.532		q>7.249
	30v35	10.294		q>7.249
Dry BC	k	df	**q**	**T**
	4	8	4.529	0.0693
	Temperature (°C)	$\lvert\bar{x}_1 - \bar{x}_2\rvert$		***Significant diff.**
	20v25	0.0306		q<0.0693
	20v30	0.0088		q<0.0693
	20v35	0.0829		q>0.0693
	25v30	0.0218		q<0.0693
	25v35	0.0524		q<0.0693
	30v35	0.0742		q>0.0693

*q-value < T-value has no significant difference

The post-hoc test reveals a significant difference in BC weight between the lowest temperature (20°C) and the highest temperature (35°C) (Table 2). This implies that large temperature variations substantially impact BC production, with lower temperatures promoting higher yield. Lower temperatures (<20°C) could increase the efficiency of BC production. Nevertheless, the reduction in BC weight observed at 25°C could potentially be linked to fluctuations in temperature occurring within the incubator. Fluctuation of temperature causes physiological stress to the bacteria; hence, it disturbs the cellular activities of the bacteria (Ivancic et al., 2013) and causes *K. sucrofermentans* to produce BC inefficiently.

The Production of Bacterial Cellulose in Different Aeration Rates

Figure 3 displays the wet and dry weight BC harvested after 7 days of fermentation with different aeration. Four aeration rates (20%, 40%, 60%, 80%) were set upon BC production by *K. sucrofermentans.* Maximum BC yield was obtained from fermentation culture with 60% aeration.

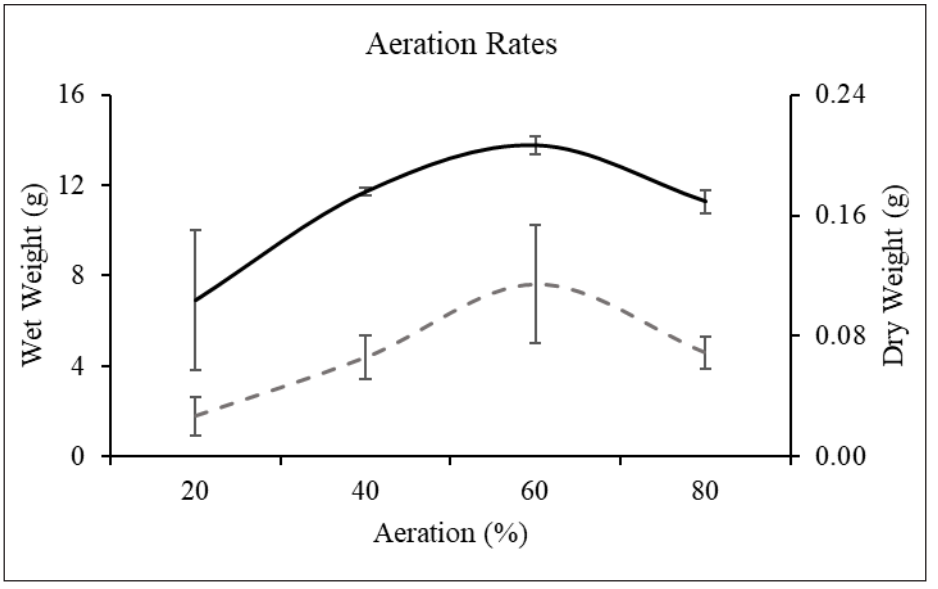


Figure 3. BC production by *K. sucrofermentans* under varying aeration rates ranging from 20% to 80%. The graph shows the weight of BC; The smooth line denotes the wet weight of BC, and the dashed line denotes the dry weight of BC

The highest wet and dry weights obtained from the fermentation culture were at 60% aeration, with approximately 13.7 g and 0.115 g, respectively. In contrast, wet BC obtained from other aeration levels ranged from 6.0 g to 11.7 g, and dry BC ranged from 0.2 g to 0.6 g—both significantly lower than the BC produced with 60% aeration. The production of BC exhibited an increase at 60% aeration but declined as aeration levels increased to 80%.

The aeration rate exhibits the least significant impact, as shown in Table 3. A notable difference in BC production is observed between 20% and 60% aeration, but this effect is primarily evident in wet BC. In contrast, the aeration rate does not have a measurable influence on the characteristics of the BC membrane once it has dried. The lack of significance observed in dry weight may be attributed to the close variance in dry BC between 20%, 40% and 80%, which were close to each other (20%, $\sigma^2 = 0.00017$; 40%, $\sigma^2 = 0.00021$; 80%, $\sigma^2 = 0.00012$). Following the principles outlined in "Understanding Analysis of Variance" by Natoli (2017), the variance within or between factors influences the statistical significance of parameters.

Low aeration reduces efficiency and limits the biosynthesis of BC by *K. sucrofermentans* while excessive aeration can prove detrimental due to oxygen saturation (Shavyrkina et al., 2021). In such instances, surplus oxygen acts as a proton acceptor, converting glucose to gluconic acid, thus reducing cellulose production (Tantratian et al., 2005). Hence, optimizing the balance between media culture and aeration is crucial for enhancing cellulose synthesis and obtaining a higher yield of BC.

Table 3
Post hoc Tukey test, comparison between the mean of each aeration rate to the production of BC

Wet BC	k	df	**q**	**T**
	4	4	5.757	6.437
	Aeration (%)	$\lvert\bar{x}_1 - \bar{x}_2\rvert$		***Significant diff.**
	20v40	4.783		q<6.437
	20v60	6.838		q>6.437
	20v80	4.357		q<6.437
	40v60	2.055		q<6.437
	40v80	0.426		q<6.437
	60v80	2.481		q<6.437
Dry BC	k	df	**q**	**T**
	4	4	5.757	0.0918
	Aeration (%)	$\lvert\bar{x}_1 - \bar{x}_2\rvert$		***Significant diff.**
	20v40	0.0389		q<0.0918
	20v60	0.0876		q<0.0918
	20v80	0.0420		q<0.0918
	40v60	0.0487		q<0.0918
	40v80	0.0031		q<0.0918
	60v80	0.0456		q<0.0918

*q-value < T-value has no significant difference

The Production of Bacterial Cellulose in Agitated Culture and Static Culture

Agitation was conducted at 50 rpm, 150 rpm, and 250 rpm; however, shaken cultures at 150 rpm and 250 rpm did not yield any BC. A comparison was made between static conditions and cultures shaking at 50 rpm, revealing that shaken cultures produced higher amounts of BC than static cultures (0 rpm) (Figure 4).

The average weight of wet and dry BC obtained from 50 rpm shaken culture was 14.97 g and 0.09 g, respectively. The average weight of wet BC gained from static culture (0rpm) was 10.75 g, while dry BC had an average of 0.07g. These results indicate that BC derived from shaken cultures at 50 rpm exhibits a higher yield compared to that from static cultures, both in wet and dry forms. There was a significant difference between each agitation (wet weight BC; p=0.00141, dry weight p=0.0199, p-*value*<0.05). Agitation helps distribute nutrients evenly and supply oxygen efficiently, which improved the synthesis of BC by *K. sucrofermentans* in a 50 rpm shaken culture (Zhou et al., 2018). Static culture has lower production compared to agitated culture due to the limited oxygen supplied to the bacteria (Ul-Islam et al., 2015). This shows that agitated culture can be used to mass-produce bacterial cellulose (Lahiri et al., 2021; Rahman et al., 2021). Nonetheless, as the shaking went higher, the production of BC was inhibited, and an intense cloudy solution

was produced. This results in failing to achieve cellulose for any cellulose above 50 rpm agitation. As Rahman et al. (2021) reported, acetic acid bacteria have more difficulties producing BC in agitated culture. Agitation during fermentation promotes the development of non-producing cellulose mutant cells (Cel-) in response to shear stress, consequently reducing their metabolic performance (Jasme et al., 2022; Raghavendran et al., 2020). A similar observation could be made in the culture of the *K. sucrofermentans* strain at higher shaking rates when it failed to produce cellulose.

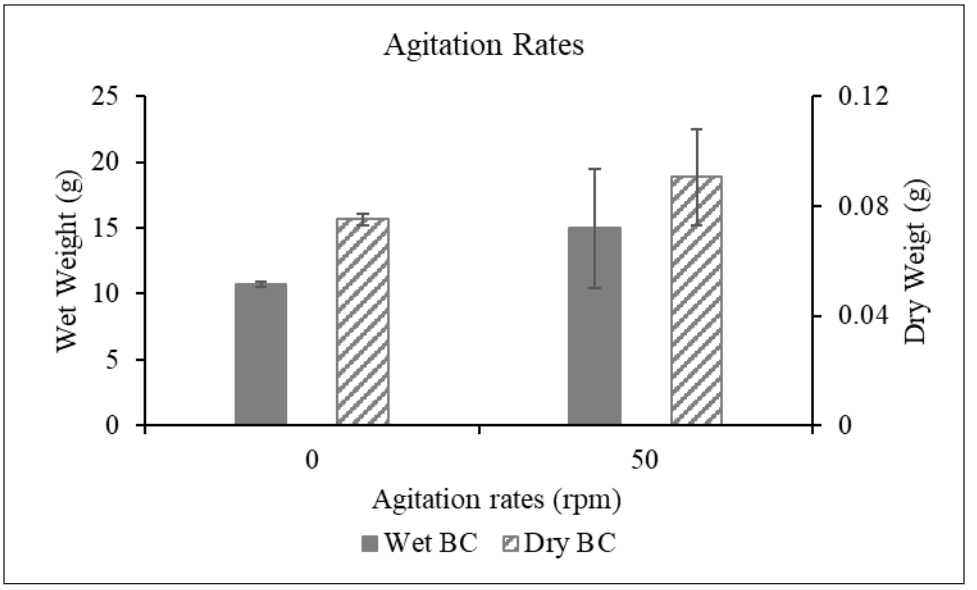


Figure 4. Production of BC by *K. sucrofermentans* in static culture and slow agitated culture. The graph shows the weight of BC; the fill bar graph denotes the wet weight of BC; and the diagonal stripes bar denotes the dry weight of BC

The Production of Bacterial Cellulose in Different Surface Areas

The surface area (SA) parameter was added to observe the effect of different areas on the efficiency of BC production by *K. sucrofermentans.* Larger surface areas yield the highest amount of BC and result in much larger BC membranes compared to other surface areas (Figure 5). The size of BC membranes across different surface areas is detailed in Table 4.

Maximum BC produced was on large SA (63.62 cm^2), obtained about 17.61 g of wet BC and 0.14 g of dry BC. Smaller SA (19.63 cm^2) yields the lowest BC at about 2.66 g for wet BC and only obtained 0.011 g for dry BC (Figure 5). The size of the BC membrane can be seen differently across the surface areas. The BC membrane was smaller when fermented in 19.63cm^2. When dried, it shrunk into much smaller pieces, as displayed in Table 4. BC production increases as surface areas increase. Larger surface areas provide more nutrient content and supply more oxygen, which improves the metabolic process of

K. sucrofermentans in producing BC (Abou-Taleb & Galal, 2018; Abusham et al., 2009). This can be seen in Table 4, where the BC membrane in a large surface area (63.63 cm^2) exhibits a thicker membrane, looking opaque compared to other surface areas of culture fermentation. The effects on the surface area are highly significant and distinctly varied (Wet BC; p=0.00047, Dry BC p=1.88E-07; p<0.05). This can be seen in Table 5.

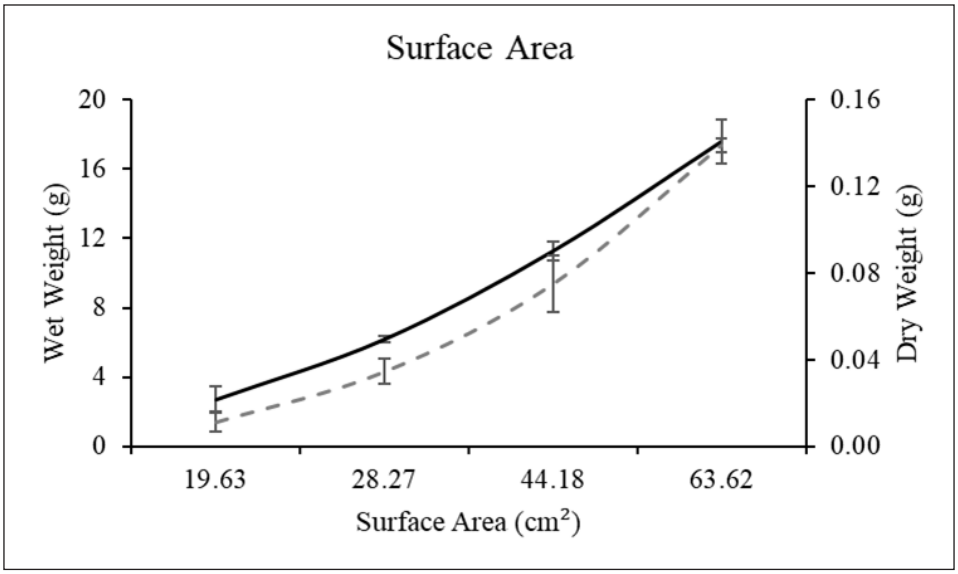
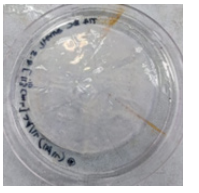
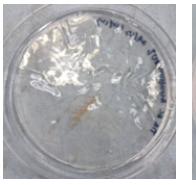
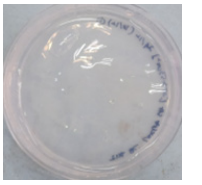
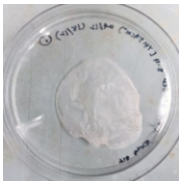
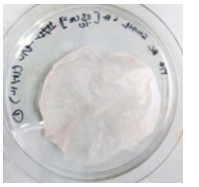
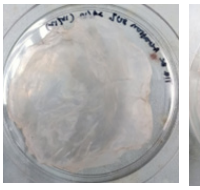
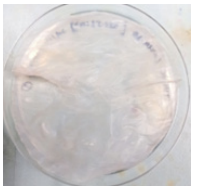


Figure 5. BC production by *K. sucrofermentans* with different surface areas from 19.63 cm^2 to 63.62 cm^2. The graph shows the weight of BC; The smooth line denotes the wet weight of BC, and the dashed line denotes the dry weight of BC

Table 4
The wet and dry membranes of BC were harvested from different surface area cultures after 7 days of fermentation

	19.63 cm^2	**28.27 cm^2**	**44.18 cm^2**	**63.62 cm^2**
Wet BC				
Dry BC				

The post-hoc test for surface area revealed a statistically significant difference in cellulose production efficiency for both wet and dry BC. This finding suggests that variations in surface area significantly impact the efficiency of cellulose production by bacteria. This suggests that BC yields vary based on surface areas. Larger surface areas demonstrate greater efficiency in mass-producing BC, providing evidence that this parameter can be further optimized for production on a larger scale in an industrial setting.

Table 5
The Post hoc Tukey test compares the mean of each surface area to the production of BC. A=19.63 cm² B=28.27 cm² C= 44.18 cm² D=63.62 cm²

Wet BC	**k**	**df**	**q**	**T**
	4	8	4.529	7.094
	Surface Area (cm²)	$\lvert\bar{x}_1 - \bar{x}_2\rvert$		***Significant diff.**
	AvB	3.497		q<7.094
	AvC	11.615		q>7.094
	AvD	14.953		q>7.094
	BvC	8.118		q>7.094
	BvD	11.455		q>7.094
	CvD	3.338		q<7.094
Dry BC	**k**	**df**	**q**	**T**
	4	8	4.529	0.0202
	Surface Area (cm²)	$\lvert\bar{x}_1 - \bar{x}_2\rvert$		*Significant diff.
	AvB	0.023		q>0.0202
	AvC	0.064		q>0.0202
	AvD	0.128		q>0.0202
	BvC	0.040		q>0.0202
	BvD	0.104		q>0.0202
	CvD	0.064		q>0.0202

*q-value < T-value has no significant difference

CONCLUSION

Physical conditions play a vital role in influencing the production of BC. This study examined various parameters to ascertain the optimal conditions for BC production. Based on the maximum yield observed across different parameters, conditions that enhance the synthesis of bacterial cellulose (BC) were identified: pH 5, temperature of 20°C, aeration rates of 60%, shaking rates at 50 rpm, and surface areas (≥63.62 cm²). Through ANOVA analysis, each parameter was found to significantly influence BC production. The physical parameters influencing BC production are crucial; failing to maintain optimal conditions diminishes bacterial activity, as evidenced by fluctuations in factors like pH and

temperature. As these factors deviate from their optimal ranges, the efficiency of cellulose synthesis declines, which can lead to lower production of cellulose. This important finding on physical characterization has the potential to significantly enhance cellulose production efficiency by *K. sucrofermentans* at an industrial scale.

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