Implementation of real-time image processing on bacterial cellulose formation using soybean-boiled wastewater with the variation of carbon sources during fermentation

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Abstract. Bacterial cellulose (BC) is produced by aerobic bacteria through oxidative fermentation in synthetic and non-synthetic mediums. Several mediums reported to be used as BC formation mediums are coconut water and soybean-boiled wastewater. Carbon sources are needed to optimize the BC formation process. Recent study has implemented a real-time image processing approach for monitoring BC formation. This study aimed to investigate the correlation between variables that influence the fermentation and to determine the kinetic model of BC formation using an image processing approach with the variation of carbon sources during the fermentation. The results showed that the correlation between fermentation time and thickness had the highest percentage for glucose, sucrose, and mannitol mediums. The kinetic observation of BC formation in the medium using glucose, sucrose, and mannitol followed the Gompertz model equation, with the medium using sucrose having the fastest rate of increase at the 44th hour, followed by the medium using mannitol at the 112th hour, and the medium using glucose at the 149th hour.

1 Introduction

Bacterial cellulose (BC) is produced by aerobic bacteria such as *Acetobacter xylinum* through oxidative fermentation in synthetic and non-synthetic mediums [1]. *Acetobacter xylinum* is a Gram-negative bacterium that ferments actively at a pH range from 3 to 7 and a temperature range from 25°C to 30°C [2]. BC retains the fundamental structure of a fibril composed of β-1→4 glucan chains with the formula $(C_6H_{10}O_5)_n$ held together by intra- and inter-hydrogen bonds [3, 4]. Chen et al. [5] stated that through biosynthesis, bacteria secrete glucan chains through pores arranged in regular lines along the long axes of the cell's cytomembrane.

The most commonly used BC formation medium is coconut water [6, 7, 8]. Furthermore, wastewater can also be used as a BC formation medium, such as liquid waste from sugarcane processing during the jaggery [9], juice from Japanese pear, orange, apple, grape, and pineapple waste [10], waste beer yeast [11], rice wine distillation wastewater [12], liquid waste from tofu production [13], and soybean-boiled wastewater from tempeh production [14]. In addition, Maloringan et al. [14] used soybean-boiled wastewater as a BC formation medium because it contains nitrogen used by bacteria as a source of nutrients during fermentation.

Wang and Zhong [15] stated that a substrate in the form of carbon sources is needed to optimize the BC formation process. There are many carbon sources reported to be used, such as sucrose [2, 6-8, 10, 16-19], glucose [2, 10-12, 16-19], fructose [2, 10, 16, 19], mannitol [2, 16-19], galactose [2, 19], maltose [2], glycerol [16, 18, 19], date syrup [17], and lactose [18]. Ruka et al. [16] stated that of the various types of carbon sources, sucrose, mannitol, and glucose are carbon sources that produce consistently high yields of cellulose, regardless of the medium composition used. Other variables affecting BC formation besides carbon and nitrogen sources include BC layer thickness [7], fermentation time [19, 20], pH [20, 21], temperature [21], and turbidity conditions [22].

The fermentation commonly takes 6-8 days [7]. Traditionally, the observation of the formation of the BC during the fermentation is carried out after the fermentation has been completed by opening the lid of the tray and lifting a layer of BC to determine the thickness. The observation determines the formation amount that occurred during the fermentation. This step aims to ascertain how much the BC has expanded as a result of the fermentation [6]. Nugroho et al. [7] revealed a new observation method using real-time image processing (RTIP). In addition, Poka et al. [23] stated that the observation using RTIP was determined through edge detection. As a result, the edge detection algorithm has successfully separated BC layer object from other objects such as medium, BC non-layer, and air space between the fermenter cover and the medium [8].

In agricultural fields, RTIP that uses large amounts of data itself has been widely applied, including being used for early detection systems on cocoa pest attacks

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[24], classification of the quality of whole plant corn silage (WPCS) for cattle feed [25], and the creation of an automatic weed control system that can distinguish weeds and plants correctly in tomatoes [26], celeries [27], potatoes [28], and lettuces [29]. There are similarities in the functions of these studies, i.e. RTIP is used as an early warning system to identify anomalies that occur in the study objects. Furthermore, RTIP can become a decision support system based on the data that has been captured.

In microbiological fields, particularly in the production of BC, the mathematical model developed from RTIP has potential applications in industry. It can serve as an early warning system for contamination during BC fermentation through the detection of anomalies in BC formation patterns and a decision support tool for determining the fermentation time required for achieving certain BC layer thickness. However, the application of RTIP in observing BC formation is still limited to coconut water medium and sucrose carbon source. Therefore, this study aimed to investigate the correlation between variables that influence the fermentation and to determine the kinetic model of BC formation using a RTIP approach with the variation of carbon sources, i.e. sucrose, mannitol, and glucose during the fermentation using soybean-boiled wastewater.

2 Material and Method

2.1 Starter Production

The organism used as a starter was *Acetobacter xylinum*. The starter created using 1.5 L coconut water, 5% glucose, 0.2% ammonium sulfate, 0.2% acetic acid, and 20% pure starter. The fermentation was carried out for 7-13 days so that the starter is ready to use.

2.2 Medium Production

The BC medium was composed of coconut water and soybean-boiled wastewater in a ratio of 70:30 with a total volume of 2.5 L [14]. The other constituent components were carbon sources (10% glucose [30], 5% sucrose [6], or 0.83% mannitol [31]), 0.2% ammonium sulfate [30], 0.2% acetic acid [30], and 20% starter [32]. All components except the starter were heated (T = 100°C; t = 30 s) and then heated at a lower temperature (T = 60°C; t = 10 m). After that, the BC medium was left until it reached room temperature. Subsequently, the starter was added to the medium. Last, it was poured into the fermenter.

2.3 Fermentation Process

The fermentation method used in this study was static and batch fermentation [6]. No additional treatment was applied during fermentation. Fermentation was carried out until the stationary phase of bacterial growth was reached. After reaching this phase, the fermentation was stopped. Later, the thickness of the BC layer was measured manually with a caliper. The purpose of manual measurement is to be used as a reference in converting pixel units into cm units.

2.4 Fermenter

The fermenter used [8] composed of acrylic material painted with a non-reflective black color on the external surface and measuring 35 cm in length, 25 cm in width, and 6 cm in height. The transparent field was created facing the USB camera with a width of 6 cm and a height of 4 cm. The fermenter's lid is made of paper that allows oxygen to pass through. An LED light is positioned in front of the fermenter to increase the contrast of the object with the camera by providing lighting assistance. The USB camera with a resolution of 640 x 480 pixels and manual focus features is positioned directly in front of the transparent field. Figure 1. shows the fermenter model used.

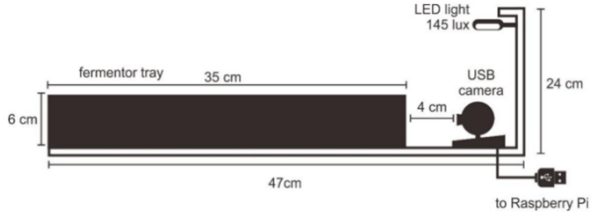


Fig. 1. Fermenter model [8].

2.5 Equipment Used

The equipment used refers to Nugroho et al. [8], which consists of Raspberry Pi 4, Arduino UNO, and four sensors, i.e. USB camera, temperature, pH, and turbidity sensor. The selection of four sensors used in this study is based on what variables are influential during fermentation, i.e. fermentation time, BC layer thickness, temperature, pH, and turbidity conditions. The USB camera sensor is used to capture BC layer thickness data which is directly connected to the Raspberry Pi 4. The temperature, pH, and turbidity sensor are positioned inside the fermentation medium at a depth of 1 cm from the bottom of the fermenter. The data obtained from temperature, pH, and turbidity sensor will be sent to the Arduino UNO, where it will be converted from analog-type data into digital-type data that the Raspberry Pi 4 can read.

Raspberry Pi 4 is a low-priced and pocket-sized computer. This board includes two USB 3.0 and two USB 2.0 ports, 40 pins, a Micro-SD card slot, two micro-HDMI ports, and WiFi and Ethernet support. The operating system is open-source and free software based on the Linux kernel called Raspbian Pi OS. The MySQL database's BC thickness calculation method and command insertion mechanism were developed using Python programming [8].

2.6 Telegram Bot

Telegram Bot can send messages automatically within a specific time range. Rianto et al. [33] reported that Telegram Bot could be used to send academic information services automatically. Trirahma [34] used

Telegram Bot as a real-time data-collecting tool for area mapping activity progress reporting.

In this study, Telegram Bot was used to monitor the progress of BC formation by sending messages containing information such as data collection time, fermentation code, BC layer thickness, temperature, pH, and BC medium turbidity. This was possible because the Telegram Bot was connected by PHP Handler to a Python script on the Raspberry Pi 4. Furthermore, the Raspberry Pi 4 was connected to the internet, and automatically the script was run every 15 minutes.

2.7 Data Collection

Nugroho et al. [8] explained that the data collection scheme starts with data transmission. The image the USB camera captures is sent to the Raspberry Pi 4 for BC thickness calculation. Simultaneously, the captured temperature, pH, and turbidity data will be sent to the Raspberry Pi 4 through the Arduino UNO. After that, all this data is entered and stored in the MySQL database installed on the Raspberry Pi 4. Concomitantly, the data is sent to the user's Telegram account. Data were collected every 15 minutes from the beginning until the stationary phase of fermentation. Figure 2. shows the data collection scheme.

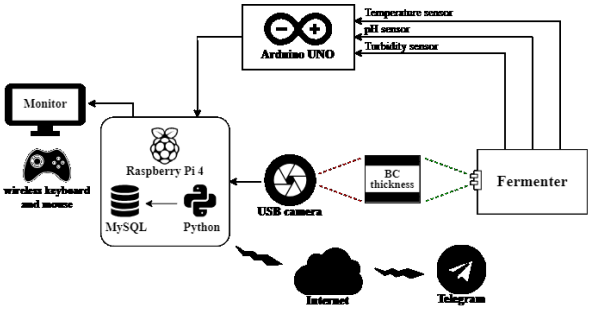


Fig. 2. Data collection scheme [8].

2.8 Statistical Analysis using R and Rstudio

R and Rstudio are used for statistical analysis [5]. The statistical analysis includes correlation, principal component, clustering, and kinetic model analysis. The R version used is 4.3.1, which can be downloaded from https://cran.r-project.org/bin/windows/base/. The GUI application uses the RStudio 2023.06.0+421 version, which can be downloaded from https://download1.rstudio.org/electron/windows/RStudio-2023.06.0-421.exe

3 Result and Discussion

3.1 Data Collection Results during BC Fermentation

The four sensors used successfully captured data on thickness, temperature, pH, and turbidity during fermentation with the medium using three different types of carbon sources. The data was then plotted into the graphs shown in Figure 3, 4, and 5.

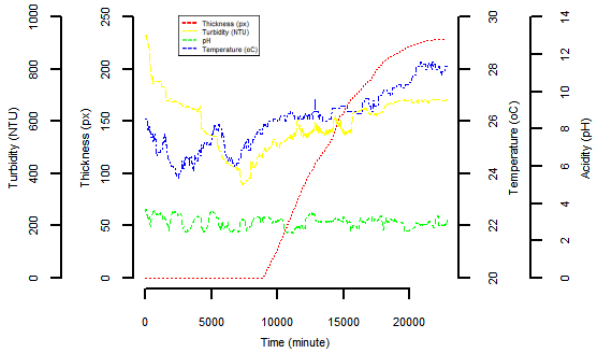


Fig. 3. Data collection result in the medium using glucose.

In the medium using glucose, as shown in Figure 3, the BC layer is visually visible in the 149th hour, and after that hour, there is an exponential increase in BC thickness until fermentation ends. The initial temperature condition before the BC layer appeared was at 24-26°C. When the BC layer became visually visible until the end of fermentation, the temperature was at 26-28°C. This increase occurs due to heat release by the inoculum, which indicates an increase in metabolic rate during the formation of the BC layer. The temperature condition is aligned with Nugroho et al. [8], although the medium used differs. Subsequently, the turbidity condition showed a significant decrease until the initial phase of the BC layer formation caused by biomass deposition in the fermenter. After that, the turbidity became stable until the end of fermentation because the inoculum increasingly secreted BC fibers, forming the BC layer. Furthermore, the pH condition was at 2-4 from the beginning of fermentation to the end. In this medium, fermentation lasted for 16 days with the final BC layer thickness of 1.08 cm.

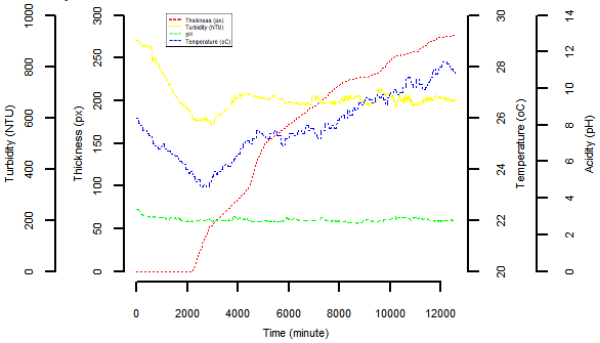


Fig. 4. Data collection result in the medium using sucrose.

In the medium using sucrose, as shown in Figure 4, the BC layer can be seen visually starting from the 37th hour, increasing exponentially until the end of fermentation. In the initial phase of pouring until before the formation of the BC layer, the temperature was at 24-26°C, and after the BC layer was formed until fermentation ended, the temperature was at 24-28°C. Hereafter, turbidity conditions in this medium have a similar pattern to those in the medium using glucose. From the initial phase to the BC formation phase, there was a decrease in turbidity, and then increased until fermentation ended. Moreover, the pH condition did not change significantly during fermentation. Nugroho et al. [8] reported similar pH results when using sucrose as a

carbon source. In this medium, fermentation lasted for 9 days with the final BC layer thickness of 1.31 cm.

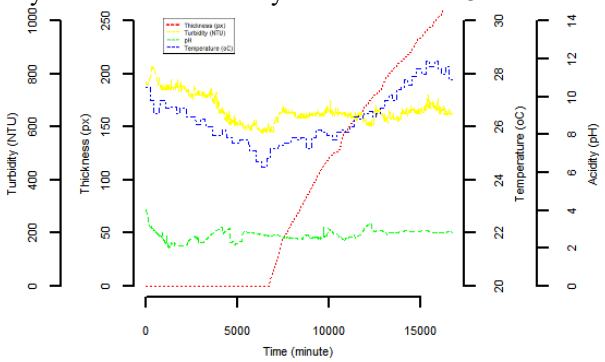


Fig. 5. Data collection result in the medium using mannitol.

In the medium using mannitol, as shown in Figure 5, the BC layer can be visually seen from the 112th hour, and the exponential increase in thickness occurs after that hour. In the phase after biomass decanting until before the formation of the BC layer, the temperature was 24-27°C, and after the formation of the BC layer until the end of fermentation, the temperature was 25-29°C. Furthermore, turbidity in the initial phase after biomass pouring decreased until the initial phase of BC layer formation and then became more stable until the end of fermentation. In addition, the pH condition was at 2-4 during fermentation. In this medium, fermentation lasted for 11 days with the final BC layer thickness of 1.27 cm.

The similarity of the pattern of temperature and turbidity changes from the use of three different carbon sources shows that the different types of carbon sources do not significantly impact the pattern of temperature and turbidity changes during fermentation. Furthermore, differences in carbon sources affect the pattern of pH changes. The medium using sucrose has the most stable pH pattern compared to the other two mediums. The medium using sucrose also produced the thickest BC layer with the fastest fermentation time compared to the other two mediums.

3.2 Correlation Analysis

Correlation analysis measures the level of relationship between a variable and another variable [35]. Analysis was performed on thickness, temperature, pH, turbidity, and fermentation time to determine which variable had the highest percentage level of relationship. Figure 6, 7, and 8 shows the results of the correlation analysis.

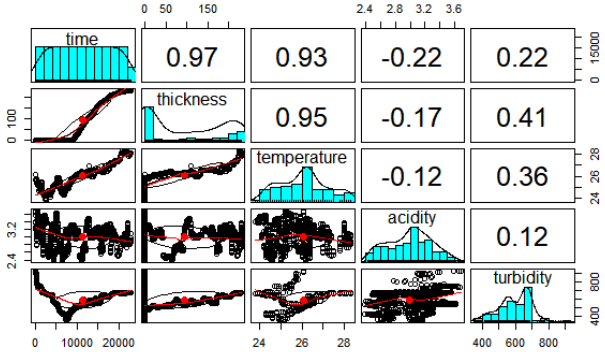


Fig. 6. Correlation analysis result in the medium using glucose.

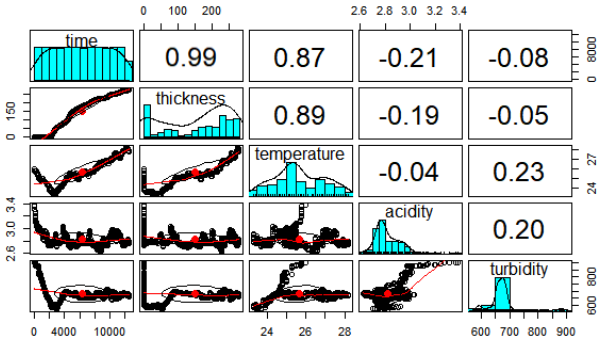


Fig. 7. Correlation analysis result in the medium using sucrose.

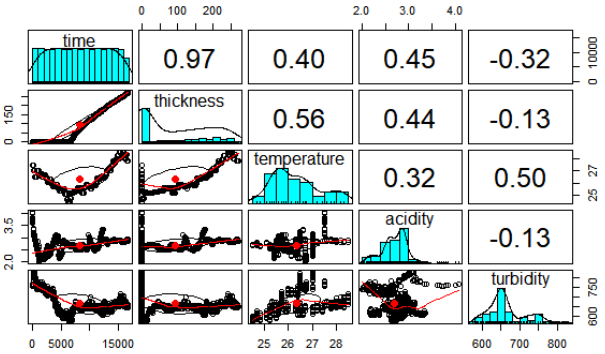


Fig. 8. Correlation analysis result in the medium using mannitol.

In the medium using glucose, as shown in Figure 6, fermentation time and thickness relationship had the highest correlation of 97%, followed by the correlation between temperature and thickness of 95%. In the medium using sucrose, fermentation time and thickness relationship had the highest correlation of 99%, followed by the relationship between temperature and thickness of 83%. In the medium using mannitol, fermentation time and thickness relationship had the highest correlation of 97%, followed by the relationship between temperature and thickness of 56%.

The results of the correlation analysis of the three mediums showed that the relationship with the highest correlation was between fermentation time and thickness. Mikkelsen et al. [19] explained that the longer the fermentation time, the more thickness of the BC layer will increase. Ruka et al. [16] also stated the same thing, regardless of the different types of carbon sources used. As a result, the length of fermentation time is highly correlated with the increase in BC layer thickness despite the different types of carbon sources used.

3.3 Principal Component Analysis

Principal component analysis (PCA) aims to reduce the variables in data to a smaller number of variables without losing the information contained in the original data [36]. Although there are fewer variables, only using the PCA data will produce the same value as using the original data. To determine it is based on the percentage of the cumulative proportion of variance. According to Jolliffe & Cadima [37], if the percentage of the cumulative proportion of variance has reached 70%, it is considered sufficient to represent the total diversity of

the data. Therefore, the PCA result data will be used for the clustering analysis.

Table 1. Principal component analysis result.

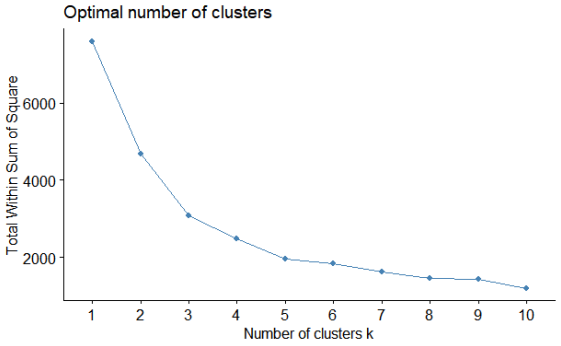
Principal Component	Cummulative Proportion Values		
	Glucose	Sucrose	Mannitol
PC1	0.59790	0.58870	0.55100
PC2	0.84500	0.90770	0.82800
PC3	0.97680	0.98693	0.97490
PC4	0.99827	0.99850	0.99865
PC5	1.00000	1.00000	1.00000

Table 1 shows that PCA resulted in five principal components, namely PC1, PC2, PC3, PC4, and PC5. The cumulative proportion value of PC1 and PC2 in the medium using glucose is 84.5%. This result means that PC1 and PC2 were able 84.5% of the data diversity. The cumulative proportion value of PC1 and PC2 in the medium using sucrose is 86.07%. This result means that PC1 and PC2 were able 86.07% of the data diversity. The cumulative proportion value of PC1 and PC2 in the medium using mannitol is 82.8%. This result means that PC1 and PC2 were able 82.8% of the data diversity. Based on the results of the three mediums, it is known that data containing only PC1 and PC2 can be used to replace the original data because that data had cumulative proportion values more than 70%. The information included by PC1 and PC2 is proven to represent the information owned by the actual data [8].

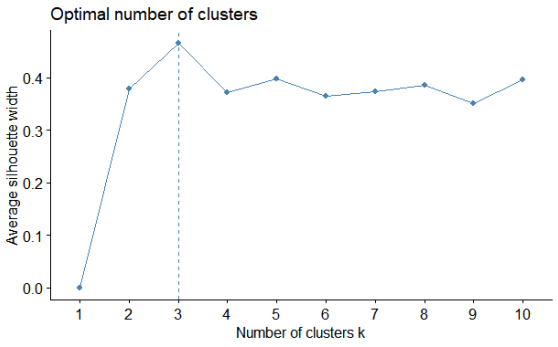
3.4 Clustering Analysis

Clustering analysis aims to divide data into groups where data in the same group have similar characteristics compared to other groups [38]. Based on the relationship between fermentation time and thickness in Figure 6, 7, and 8, clustering analysis was performed on the data during fermentation. Analysis begins with determining the optimal number of clusters.

The methods used for the determination were the within-cluster-sum of square (wss) and silhouette [39]. The purpose of using two different methods is to compare the results of both so that the optimal number of clusters obtained is more accurate. Graphs of the clustering analysis results are shown in Figures 9, 10, and 11.

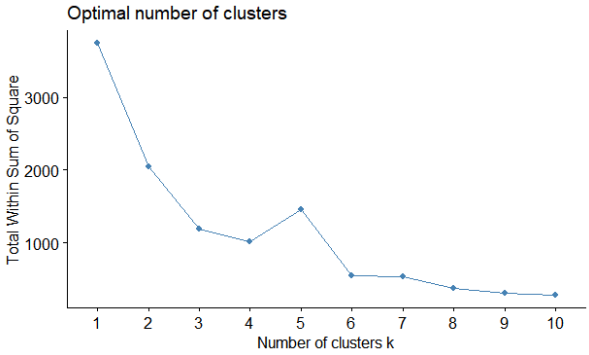


(a)



(b)

Fig. 9. The optimal cluster number determination result using the (a) wss and (b) silhouette methods in the medium using glucose.



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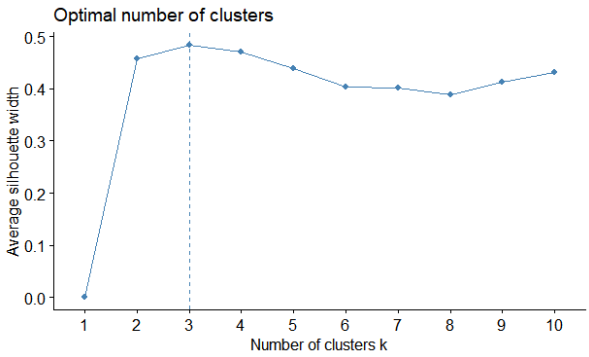
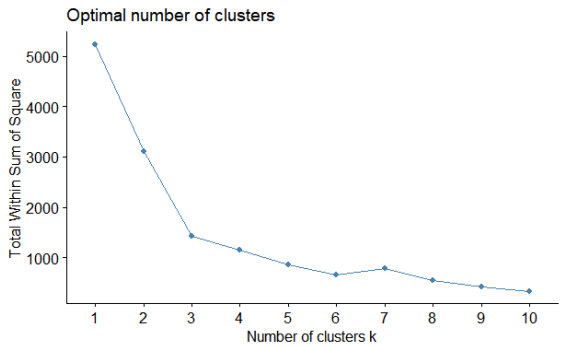
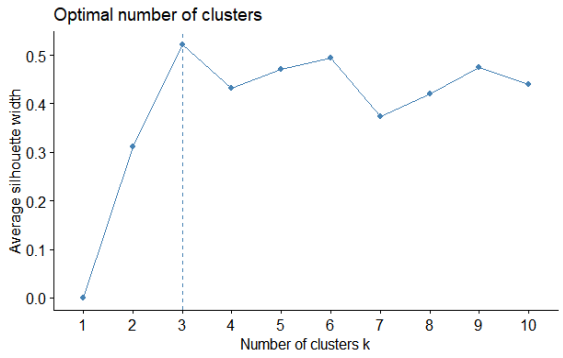


(b)

Fig. 10. The optimal cluster number determination result using the (a) wss and (b) silhouette methods in the medium using sucrose.



(a)



(b)

Fig. 11. The optimal cluster number determination result using the (a) wss and (b) silhouette methods in the medium using mannitol.

As a result, in the medium using glucose, shown in Figure 9, the optimal number of clusters produced by both methods is three. In the medium using sucrose, shown in Figure 10, the optimal number of clusters produced by both methods is three. In the medium using mannitol, shown in Figure 11, the optimal number of clusters produced by both methods is three. These results indicate that the different methods of the optimal number of clusters determination do not affect the results of the optimal number of clusters.

After the optimal number of clusters is determined, the data groups are mapped into the graph as shown in Figure 12, 13, and 14. Data group mapping is done to determine the position of each data group.

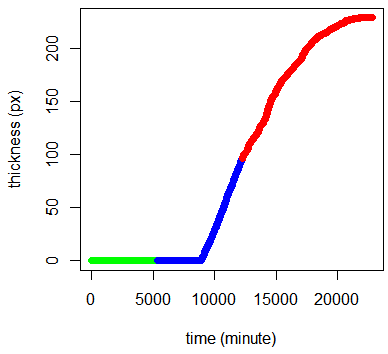


Fig. 12. Data group mapping in the medium using glucose.

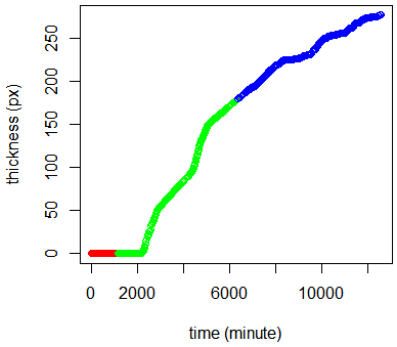


Fig. 13. Data group mapping in the medium using sucrose.

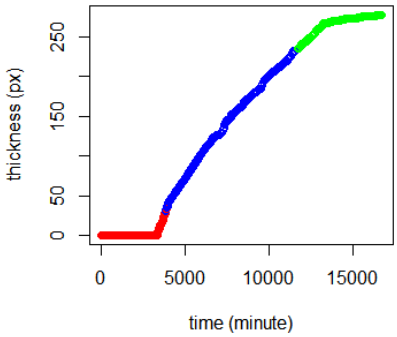


Fig. 14. Data group mapping in the medium using mannitol.

In the medium using glucose, as shown in Figure 12, the first cluster lasted until the 84th hour. The second cluster took place from the 85th until the 202nd hour. The third cluster took place at the 203rd hour until the end of fermentation. In the medium using sucrose, as shown in Figure 13, the first cluster lasted until the 18th hour. The second cluster took place from the 19th until the 115th hour. The third cluster took place at the 116th hour until the fermentation ended. In the medium using mannitol, as shown in Figure 14, the first cluster lasted until the 67th hour. The second cluster lasted from the 68th to the 204th hour. The third cluster lasted from the 205th hour until the end of fermentation.

3.5 Kinetic Model Determination of the BC Formation

After the optimal number of clusters in the medium using glucose, sucrose, and mannitol was obtained, an equation model was determined to define the BC layer's formation rate. The selection of the equation model is based on the highest R^2 value [8]. Therefore, the Gompertz model was chosen because it has been commonly used to describe the growth of animals, plants, and bacteria [40]. According to Oliviera Zardin et al. [41], this model is able to provide different biological interpretations relating to initial conditions or growth rates.

Table 2. Iteration, coefficients, and R^2 values as a result of the Gompertz equation.

Results		Carbon Sources		
		Glucose	**Sucrose**	**Mannitol**
Start Iteration Values	a	600	600	600
	b	1	1.2	1
	c	0.05	0.05	0.05
Coefficient Values	a	2.372e+02	0.027e+04	2.853e+02
	b	5.779e+01	6.492e+00	2.017e+01
	c	3.330e-04	4.359e-04	3.092e-04
R^2 Value		0.9987	0.9928	0.9948

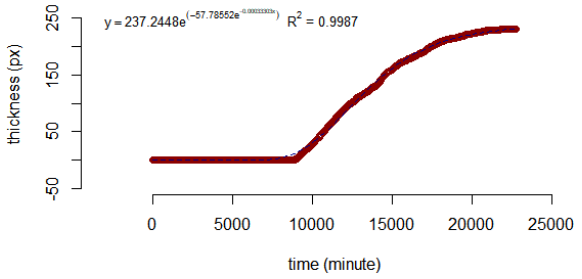


Fig. 15. Gompertz model plot of the medium thickness using glucose.

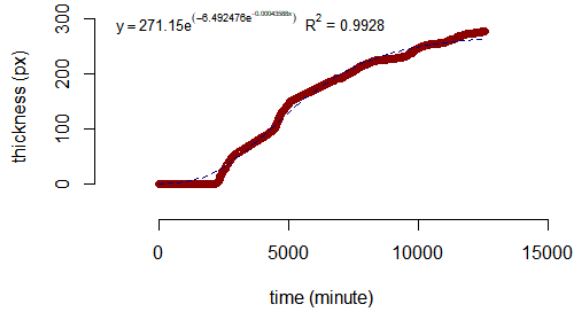


Fig. 16. Gompertz model plot of the medium thickness using sucrose.

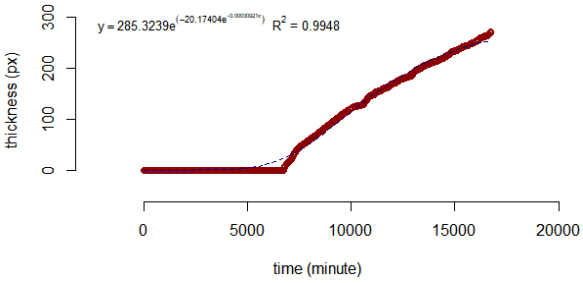


Fig. 17. Gompertz model plot of the medium thickness using mannitol.

Table 2. shows the start iteration values, the coefficient values, and the R^2 value of the Gompertz model of the medium using glucose, sucrose, and mannitol. Figure 15, 16, and 17 shows the plot of the Gompertz model used to measure BC layer thickness during fermentation. Based on the calculations that had been done, the Gompertz model is appropriate to define the BC layer's formation rate.

4 Conclusion

The correlation between fermentation time and thickness had the highest percentage in the medium using glucose, sucrose, and mannitol. The rate of BC layer formation with medium using glucose, sucrose, and mannitol followed the Gompertz model, with the medium using sucrose having the fastest rate of increase at the 44th hour, followed by the medium using mannitol at the 112th hour, and the medium using glucose at the 149th hour. Therefore, the mathematical model can serve as a reference for the future development of an early warning system and a decision support tool in the BC production industry.

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