

An Evaluation of Fermentation Period and Discs Rotation Speed of Rotary Discs Reactor for Bacterial Cellulose Production

(Penilaian Tempoh Penapaian dan Kelajuan Putaran Cakera melalui Reaktor Cakera Berputar untuk Pengeluaran Selulosa Bakteria)

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ABSTRACT

Acetobacter xylinum strains are known as efficient producers of cellulose. *A. xylinum* is an obligate aerobic bacterium that has an oxygen-based metabolism. The dissolved oxygen (DO) concentration in a rotary discs reactor (RDR) is one of the most important factors that need to be observed during the cellulose synthesis by these bacteria. In this study, the effects of different discs rotation speed (5, 7, 9 and 12 rpm) and fermentation period (3, 4, 5 and 6 days) on the DO concentration and production of bacterial cellulose in a 10-L RDR were examined. The highest yield was obtained at 7 rpm with a total dried weight of 28.3 g for 4 days fermentation. The results showed that the DO concentration in the 10-L RDR increased in the range of 13 to 17% with increasing of discs rotation speed from 7 to 12 rpm. However, fermentation with high discs rotation speed at 12 rpm reduced the bacterial cellulose production. Analysis of data using Statistica 8.0 showed a high coefficient of determination value ($R^2 = 0.92$). In conclusion, discs rotation speed gave more significant effect on the DO concentration and production of bacterial cellulose in 10-L RDR compared to fermentation period. This was further combined with synergistic effect from sufficient consumption of oxygen for the enhanced production of bacterial cellulose and providing the controlled environment for encouraging bacterial growth throughout the fermentation process.

Keywords: *Acetobacter xylinum*; bacterial cellulose; discs rotation speed; dissolved oxygen; fermentation period; rotary discs reactor (RDR)

ABSTRAK

Strain *Acetobacter xylinum* dikenali sebagai pengeluar selulosa yang cekap. *A. xylinum* adalah bakteria aerobik yang mempunyai metabolisme berasaskan oksigen. Kepekatan oksigen terlarut (DO) di dalam reaktor cakera berputar (RDR) adalah salah satu faktor terpenting yang perlu diperhatikan semasa pengeluaran selulosa oleh bakteria ini. Dalam kajian ini, kesan daripada kelajuan putaran cakera yang berbeza (5, 7, 9 dan 12 rpm) dan tempoh penapaian (3, 4, 5 dan 6 hari) terhadap kepekatan oksigen terlarut (DO) dan jumlah pengeluaran selulosa di dalam 10-L RDR telah dikaji. Hasil tertinggi telah diperolehi pada kelajuan putaran cakera 7 rpm dengan jumlah berat kering sebanyak 28.3 g selama 4 hari penapaian. Keputusan juga menunjukkan bahawa kepekatan oksigen terlarut (DO) di dalam 10-L RDR meningkat dalam julat 13 hingga 17% dengan peningkatan kelajuan putaran antara 7 hingga 12 rpm. Walau bagaimanapun, penapaian dengan kelajuan cakera yang tinggi iaitu pada 12 rpm menyebabkan pengeluaran selulosa berkurangan. Analisis data menggunakan Statistica 8.0 menunjukkan pekali nilai penentuan ($R^2 = 0.92$) yang tinggi. Kesimpulannya, kelajuan putaran cakera memberikan kesan yang lebih besar terhadap kepekatan oksigen terlarut dan penghasilan selulosa di dalam 10-L RDR berbanding tempoh penapaian. Ini seterusnya digabungkan dengan kesan sinergistik daripada penggunaan oksigen yang mencukupi bagi meningkatkan pengeluaran selulosa dan menyediakan persekitaran terkawal untuk menggalakkan pertumbuhan bakteria sepanjang proses penapaian.

Kata kunci: *Acetobacter xylinum*; kelajuan putaran cakera; oksigen terlarut; reaktor cakera berputar (RDR); selulosa bakteria; tempoh penapaian

INTRODUCTION

Bacterial cellulose is an exopolysaccharide produced by various species of bacteria, such as those of the genera *Gluconacetobacter* (formerly *Acetobacter*), *Agrobacterium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina* and *Salmonella* (Shoda & Sugano 2005). Bacterial cellulose production by *Acetobacter xylinum* was first reported in 1886 by A.J. Brown (Brown 1886). The most important culture conditions for bacterial

cellulose production are cultivation method, carbon source, nitrogen source, pH, concentration of dissolved oxygen (DO), temperature and fermentation period (Chawla et al. 2008; Jung et al. 2007; Zahan et al. 2014).

Bacterial cellulose is relatively expensive to produce (Jung et al. 1999). One way to reduce the production cost is to use cheap and readily available substrates. Unconventional feedstock from renewable resources and waste streams have been investigated (Zeng et al. 2011).

In the current research, pineapple waste was used as a fermentation medium for bacterial cellulose production in a 10-L RDR. Pineapple waste is a good source of nutrient for microorganisms due to the high carbon source and other essential elements for their survival (Zahan et al. 2014). In addition, according to the Malaysian Pineapple Industry Board, Malaysia produces a large quantity of solid and liquid pineapples wastes. The main objective of this research was to evaluate the effects of discs rotation speed (rpm) and fermentation period (days) towards the concentration of dissolved oxygen (DO) in order to enhance the production of bacterial cellulose by *A. xylinum* 0416. This was done in a 10-L RDR by utilizing pineapple waste as a medium

A. xylinum is an obligate aerobic bacterium that requires oxygen for their aerobic respiration. Oxygen also crucial for the bacteria to oxidize substrates such as glucose into energy and convert the glucose into bacterial cellulose. During the fermentation process, the bacterial cell obtains the oxygen at the air-liquid interface where bacterial cellulose was produced (Krystynowicz et al. 2005). Oxygen supply was considered as a limiting factor in their growth and bacterial cellulose formation. The DO concentration gave limitations to bacterial cellulose yield. Too high DO concentration increased the level of gluconic acid and leads to a rise of medium acidity. While too low DO concentration reduces the production rate due to insufficient oxygen source for bacterial growth (Jung et al. 1999). It was reported that bacterial cellulose production rate were proportional to oxygen transfer rate and oxygen transfer coefficient (Ougiya et al. 1997).

In order to enhance the bacterial cellulose production in 10-L RDR, the effect of fermentation period for batch culture system needs to be studied. To date, there were limited published data on the effects of fermentation period on bacterial cellulose production in 10-L RDR. In batch culture system, depleted sources of nutrients will reduce the number of cells in the medium throughout fermentation process thus reduce the production rate. Therefore, determination of fermentation period (days) towards the concentration of DO in 10-L RDR was also crucial for enhancing the bacterial cellulose production by *A. xylinum* 0416.

MATERIALS AND METHODS

EXPERIMENTAL SETUP USING 10-L RDR

Preparation and experimental setup for fermentation using 10-L RDR followed a method proposed by Pa'e et al. (2011) and Zahan et al. (2015, 2014).

PREPARATION AND MAINTENANCE OF INOCULUM *A. XYLINUM* 0416

Preparation and maintenance of inoculum *A. xylinum* 0416 in a 400 mL of liquid pineapple waste followed a method published by Zahan et al. (2015).

PREPARATION OF FERMENTATION MEDIUM

Preparation of fermentation medium using a 4 L liquid pineapple waste followed a method proposed by Zahan et al. (2015).

DETERMINATION OF FERMENTATION PERIOD

Four litre fermentation medium was prepared as mentioned by Zahan et al. (2015). Then 10% (v/v) of *A. xylinum* 0416 inoculum was poured aseptically into the fermentation medium. After that, the fermentation medium was poured aseptically into a sterilized 10-L RDR. The discs rotation speed was set at 7 rpm. The medium was left at $28\pm 1^\circ\text{C}$ for 3 days for fermentation. The concentration of DO in 10-L RDR was recorded throughout the experiment every 24 h using a DO probe (Oxy-Check, Hanna Instrument, Romania). During fermentation, 20 mL of sample was taken out from the fermentation medium every 24 h. After 3 days, the bacterial cellulose produced was harvested and the experiment was repeated twice for different fermentation periods, which were 4, 5 and 6 days (Zahan 2014).

DETERMINATION OF DISCS ROTATION SPEED

Four litre fermentation medium was prepared as mentioned by Zahan et al. (2015). Then 10% (v/v) of *A. xylinum* 0416 inoculum was poured aseptically into the fermentation medium. After that, the fermentation medium was poured aseptically into a sterilized 10-L RDR. The discs rotation speed was set at 5 rpm. The medium was left at $28\pm 1^\circ\text{C}$ for 4 days for fermentation. The concentration of DO in 10-L RDR was recorded throughout the experiment every 24 h using a DO probe (Oxy-Check, Hanna Instrument, Romania). During fermentation, 20 mL of sample was taken out from the fermentation medium every 24 h. After 4 days, the bacterial cellulose produced was harvested and the experiment was repeated twice for different discs rotation speeds, which were 7, 9 and 12 rpm (Zahan 2014).

MEASUREMENT OF DRIED WEIGHT OF BACTERIAL CELLULOSE

The measurement of the dried weight of bacterial cellulose produced followed a method published by Zahan et al. (2015).

COLONY FORMING UNIT (CFU) TEST

Colony forming unit test for the determination of *A. xylinum* 0416 growth followed a method proposed by Zahan et al. (2015).

DETERMINATION OF SUGAR CONTENT USING REFRACTOMETER

The amount of sugar content in the fermentation medium was determined using refractometer (Gen-Purpose, Bellingham and Stanley, United Kingdom). About 3 mL of distilled water was placed on the stainless steel prism

dish of refractometer using a dropper to calibrate the refractometer. After that, 3 mL of fermentation medium sample obtained during fermentation process was placed on the prism dish of the refractometer using a dropper. The values of sugar content were recorded in a percentage of Brix. The exact values of sugar content in the fermentation medium were determined using a glucose standard curve (Zahan 2014).

STATISTICAL ANALYSIS

The data obtained was statistically analyzed using Statistica (for Windows 8.0 software, StatSoft, Inc. 1984-2007) to determine the production performance and obtain the equation for bacterial cellulose production in 10-L RDR (Zahan et al. 2014). Statistical significant was evaluated using the Analysis of Variance (ANOVA) and $p < 0.05$ (probability value) was considered as significant (Quoc et al. 2012). The variables (independent factors) used in this study were: X_1 is discs rotation speed (rpm) and X_2 is fermentation period (days) and central composite design (CCD) approach was selected. In order to evaluate the experimental results, the response factors were fitted with a second-order model in the form of quadratic polynomial (1);

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2, \quad (1)$$

where Y is the predicted response (bacterial cellulose production in 10-L RDR) used as dependent factor; X_i ($i = 1$ and 2) are the independent factors; β_0 is the intercept coefficient; and β_1 , β_2 , and β_{ij} ($i = 1$ and 2 ; $j = 1$ and 2) are the model coefficients, respectively.

RESULTS AND DISCUSSION

FERMENTATION PERIOD FOR BACTERIAL CELLULOSE PRODUCTION IN 10-L RDR

As shown in Figure 1, the production of bacterial cellulose in 10-L RDR was highest at 4 days fermentation period

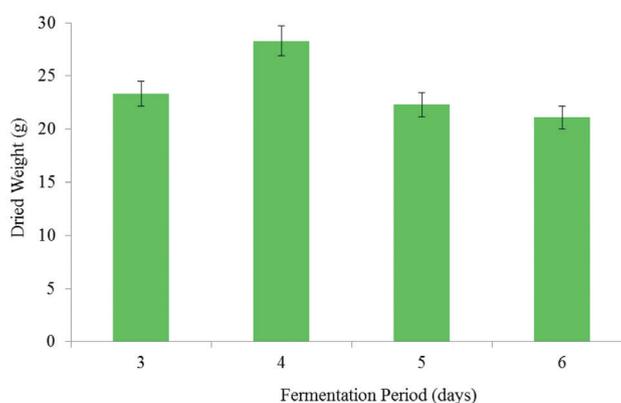


FIGURE 1. Dried weight of bacterial cellulose produced *versus* fermentation period at 7 rpm

with a total dried weight of 28.3 g. The results showed that by prolonging the fermentation period from 4 to 6 days, bacterial cellulose production reduced by about 25.5%. Bacterial cellulose production decreased with increasing fermentation time due to the reduction of bacteria viability in the fermentation medium (Figure 2). The figure illustrates the relationship between fermentation period and the number of *A. xylinum* 0416 in the fermentation medium with the dried weight of bacterial cellulose produced. It shows that the number of *A. xylinum* 0416 in the fermentation medium decreased to about 30% when the fermentation period increased from 2 to 6 days, resulted in about 25.5% declined in bacterial cellulose production. The factors that affect the bacteria viability in the fermentation medium were further studied. The first studied item was the concentration of DO in 10-L RDR. Figure 3 shows the link between the concentration of DO in 10-L RDR and *A. xylinum* 0416 growth in the fermentation medium. When fermentation period increased, the concentration of DO in 10-L RDR showed a decreasing trend. As a result, the number of *A. xylinum* 0416 also decreased. During fermentation, bacteria used oxygen for bacterial cellulose synthesis and their growth. As there was no other source of oxygen supply into the fermentation medium while the viscosity increased, the concentration of DO declined (below 10%) and *A. xylinum* 0416 could not survive for prolonged fermentation period. The results were in agreement with Chao et al. (2001) who reported that bacterial cellulose production increases with constant cell growth at 10 to 15% of DO concentration.

Theoretically, the production of bacterial cellulose in 10-L RDR will be influenced by the amount of nutrients available in the fermentation medium throughout the fermentation process. It is because RDR uses the concept of batch culture system. In batch culture system, the most required materials were loaded before the process started and removed at the end, which means that no material was added during fermentation except for pH control solutions. Thus, by prolonging the fermentation period in 10-L RDR, the nutrients primarily carbon source that is glucose will

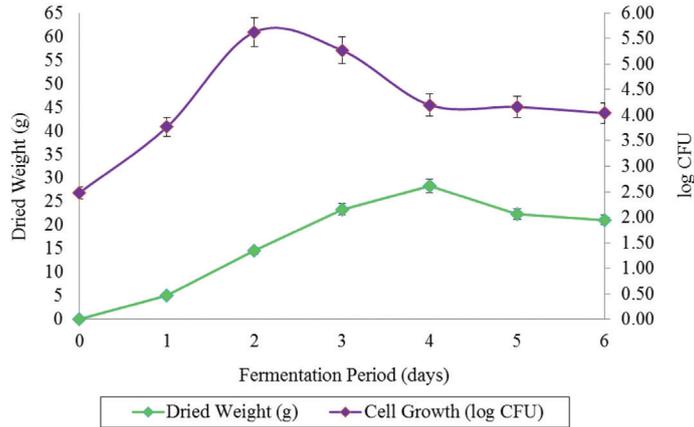


FIGURE 2. Log CFU of *A. xylinum* 0416 and dried weight of bacterial cellulose in 10-L RDR versus fermentation period at 7 rpm

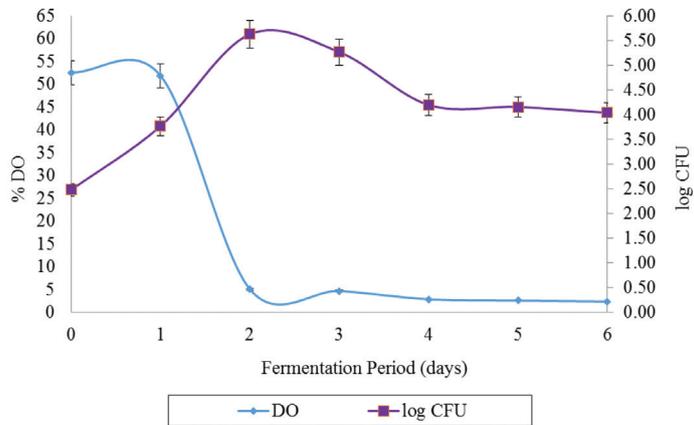


FIGURE 3. Log CFU of *A. xylinum* 0416 and concentration of dissolved oxygen (DO) in 10-L RDR versus fermentation period at 7 rpm

decrease and finally reduced the number of bacterial viability in the fermentation medium. However, in this research, it was found that the amount of glucose in the fermentation medium during the 6th day of fermentation

was still high (30.7 g/L) as shown in Figure 4. The result proved that the amount of glucose in the fermentation medium does not influence the reduction of bacteria viability in 10-L RDR, which affected the production yield.

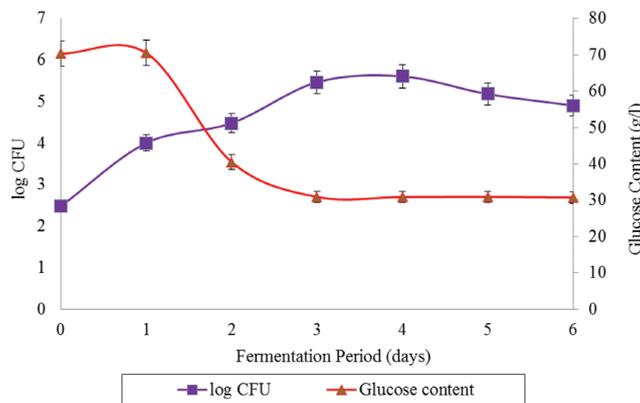


FIGURE 4. Concentration of glucose and Log CFU of *A. xylinum* 0416 in 10-L RDR versus fermentation period at 7 rpm

Obviously, the concentration of DO has a greater effect on bacteria viability compared to the amount of glucose during the prolonged fermentation period.

Other than that, the bacteria immobility could be another factor that might lead to the decreased number of bacteria viability in the fermentation medium. When the formation of bacterial cellulose film increased at the discs surface, some bacteria were entrapped in the film that finally reduced their activity and viability (Fontana et al. 1990).

DISCS ROTATION SPEED FOR BACTERIAL CELLULOSE PRODUCTION IN RDR

Figure 5 shows the dried weight of bacterial cellulose obtained at different discs rotation speed (rpm). The graph illustrates the decreased of bacterial cellulose production with increased of discs rotation speed (rpm). The highest bacterial cellulose production was obtained at low discs rotation speed (7 rpm) with the total dried weight of 28.3 g. It was perhaps related to the factor of aeration. According to previous reports (Kim et al. 2007; Krystynowicz et al. 2002; Pa'e et al. 2011), high discs rotation speed creates a harsh environment for bacteria, while providing oxygen supply in the reactor. Therefore, bacterial cellulose production declines. In this case, a discs rotation speed more than 7 rpm could be considered as high as it was harsh on bacteria activity.

Another factor that leads to the decrease in bacterial cellulose production at high discs rotation speed (12 rpm) was the creation of shear stress by the discs rotation. The shear stress could disturb the arrangement process of the crystalline fibril structure of the bacterial cellulose (Suwannapinunt et al. 2007). At low discs rotation speed (7 rpm), the attachment of bacterial cellulose on the discs surface was more organized with neat cellulose fibrils arrangement compared to that of high discs rotation speed (12 rpm) as shown in Figure 6. These results proved that the discs rotation speed affected the arrangement of bacterial cellulose fibrils. These findings were in agreement with Ruka et al. (2012) who reported that the shear stress produced by the discs rotation and agitation of the cultures does not allow strong binding of bacterial cellulose to the discs surface. Thus, the bacterial cellulose tended to lose their attachment from the discs surface and reduced the production yield as the fermentation period increased.

Meanwhile, bacterial cellulose production decreased with increasing discs rotation speed even though the concentration of DO in 10-L RDR increased as listed in Table 1. The results showed that during the second day of fermentation in 10-L RDR, the concentration of DO in the middle and bottom spaces of 10-L RDR varied at different discs rotation speeds. By increasing the discs rotation speed from 7 to 12 rpm, the concentration of DO in 10-L RDR was increased. However, it led to the

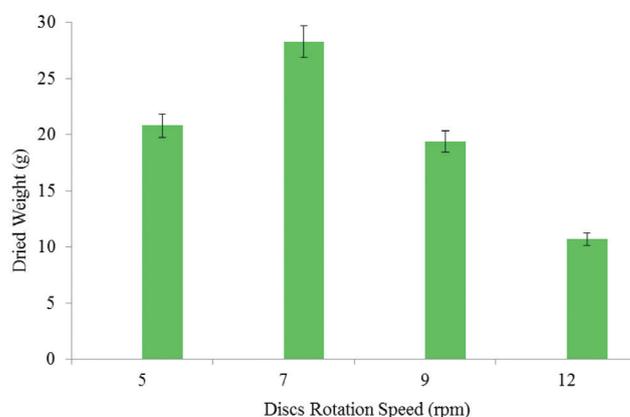


FIGURE 5. Dried weight of bacterial cellulose produced versus discs rotation speed (rpm) for 4 days fermentation

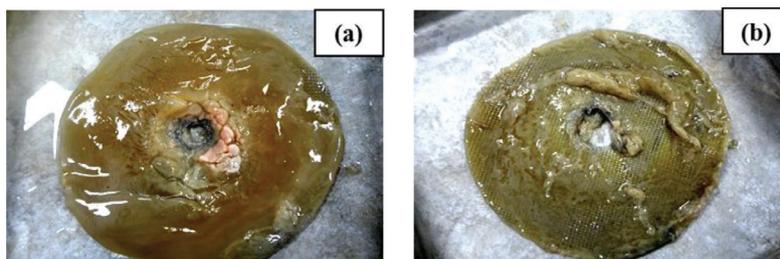


FIGURE 6. Bacterial cellulose produced in 10-L RDR at different discs rotation speed (a) 7 rpm and (b) 12 rpm

TABLE 1. Concentration of dissolved oxygen (DO) in 10-L RDR at different discs rotation speed during the second day of fermentation

Discs rotation speed (rpm)	Percentage of dissolve oxygen (%)			Final dried weight (g)
	Headspace	Middle	Bottom	
7	72.5	5.2	5.1	22.75
9	72.5	5.8	5.3	19.4
12	72.8	6.1	5.8	10.7

reduction of bacterial cellulose production. According to Kouada et al. (1997), in an excessive oxygen condition, bacterial cellulose production was decreased due to loss of substrate through direct oxidation. Hwang et al. (1999) reported the same outcome where the bacterial cellulose production decreased at high DO tension (up to 15%) in fermentation with controlled DO concentration. It was due to the high viscosity of the fermentation medium, making the controlled mixing and agitation impossible. Apparently, the DO concentration increased with increasing of discs rotation speed, but too high discs rotation speed (12 rpm) resulted in decreased of bacterial cellulose production in 10-L RDR.

Another factor that could explain this phenomenon was the change that occurred on the stability of the bacteria. According to Hestrin and Schramm (1954) and Krystynowicz et al. (2002), although continuous agitation and aeration of oxygen in a bioreactor solved many problems in static culture, the production of bacterial cellulose tend to decline due to spontaneous appearance of *Cel* mutants (cellulose nonproducer). Therefore, a strong shear stress from stirring caused genetic instability of *A. xylinum* 0416 and developed a mutant strain. Also, under agitated culture medium that supplies continuous uniform and sufficient aeration, bacteria growth is preferred instead of bacterial cellulose formation, resulting in the domination of *Cel* in the culture (Hwang et al. 1999; Kim et al. 2007). The results explained in a 10-L RDR the discs rotation speed need to be maintained at 7 rpm to provide better aeration and enough DO for the bacteria to growth without disturbing the formation of bacterial cellulose. The balance will avoid stress and help *A. xylinum* 0416 to adapt naturally and quickly to the fermentation conditions.

STATISTICAL ANALYSIS

The results were analyzed in Statistica 8.0 to visualize the effect of independent factors on the response. The quadratic model (2) expressed an empirical relationship between the response and the independent variables is;

$$Y = -10.74 + 7.54X_1 - 0.54X_1^2 + 4.47X_2 - 0.60X_2^2, \quad (2)$$

where Y is dried weight of bacterial cellulose; X_1 is discs rotation speed (rpm); and X_2 is fermentation period (days). The actual (observed) values for the combinations effects of all factors (discs rotation speed and fermentation period)

were used to assess the model responses in terms of specific numerical values in addition to the statistical evaluation of the model. The plot of predicted versus observed (actual) values for dried weight of bacterial cellulose was shown in Figure 7 with $R^2 = 0.92$. It indicated a high adequacy of the proposed model and the empirical model could not explain only 8.00% of the total variation. It also expresses a good enough quadratic fits to navigate the design space. Joglekar and May (1987) suggested that R^2 should be at least 0.80 for a good fit of a model. The R^2 value (0.92) obtained from this study was higher than 0.80, indicating that the regression models explained the process well. Thus, the model developed in this study for predicting the production of bacterial cellulose was considered to be satisfactory.

The P values were used to evaluate the significance of the model and the P values greater than 0.10 indicate the models were not significant. Here the analysis of the regression model demonstrates that the model is significant as evident from the very low probability value ($P \leq 0.000540$). Besides, the effects of discs rotation speed ($P \leq 0.022945$) was more significant compared to the fermentation period ($P \leq 0.38388$). The Pareto chart was obtained to highlight the category of factors with the highest frequency (Figure 8). From Figure 8, discs rotation speed showed the highest frequency that impacts the production of bacterial cellulose in 10-L RDR followed by the fermentation period.

CONCLUSION

As conclusions, to enhance the production of bacterial cellulose, the 10-L RDR must be operated at 7 rpm for 4 days fermentation period. The production yield decreased with increased of discs rotation speed and extended fermentation period due to some factors i.e. reduction in bacteria viability, lack of DO concentration in the fermentation medium, spontaneous appearance of *Cel* mutants (cellulose non-producer) and loss of substrate through direct oxidation in excessive oxygen condition, unavoidable entrapment of bacteria in the cellulose film layers and creation of shear stress and harsh environment by the rotation of discs. Statistical analysis also showed that the model was significant and discs rotation speed gave more significant effect to the production of bacterial cellulose in 10-L RDR compared to fermentation period.

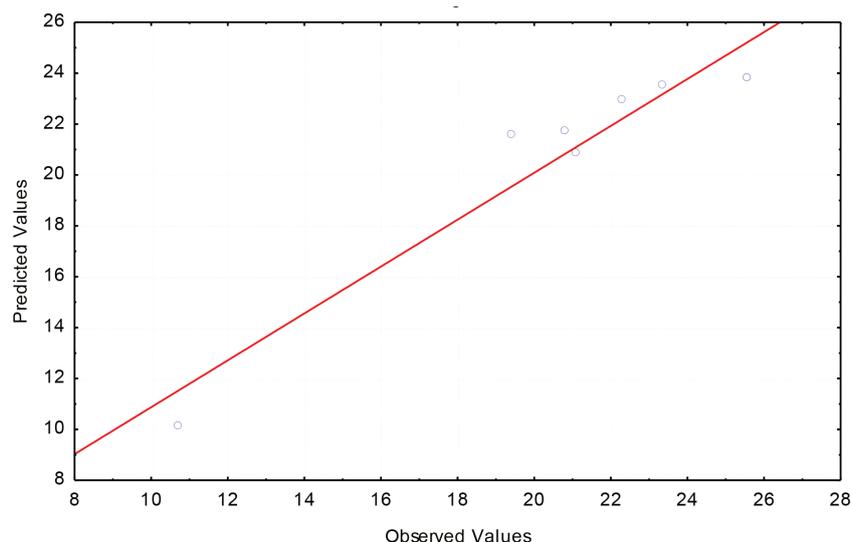


FIGURE 7. Predicted versus observed (actual) values

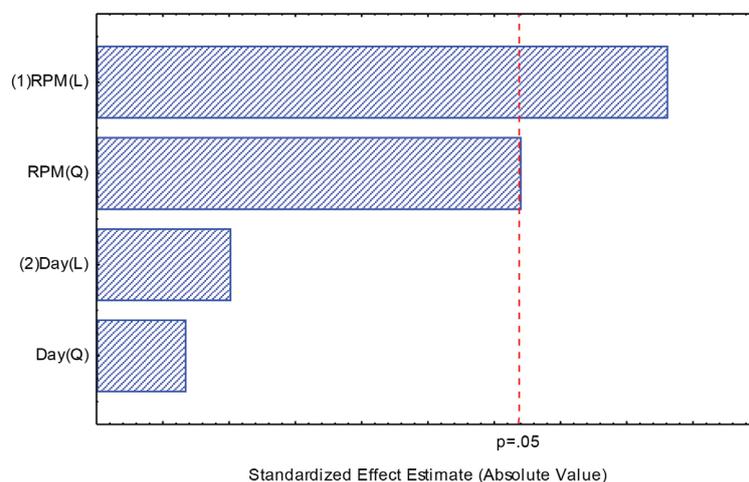


FIGURE 8. Pareto chart

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