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Influences of some ecological factors on bacterial cellulose (BC) membrane forming process in *Spirulina* medium

Ảnh hưởng của một số yếu tố sinh thái tới quá trình tạo màng bacterial cellulose (BC) trên môi trường tảo xoắn Spirulina

Research article

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Formed by a kind of bacteria called *Gluconacetobacter*, bacterial cellulose (biocellulose, BC) membrane, compared to cellulose from plants, has superior properties for the strength, toughness, durability and elasticity. The subjects of this study are bacteria being able to produce Bacterial cellulose in *Spirulina* medium. The study aims to investigate the influences of some ecological factors on the Bacterial cellulose membrane forming process in *Spirulina* medium, and then find out appropriate nutritional media and conditions for the fermentation in Bacterial cellulose forming process. The study has some major findings: (1) Select two strains of bacteria: *Gluconacetobacter xylinus* T_6 and *Gluconacetobacter xylinus* T_9, which prove to be capable of producing cellulose membrane to be used in making nourishing face masks for its thinness, smoothness, toughness and uniformity; (2) Find out the appropriate medium for the formation of Bacterial cellulose membrane, including $(NH4)_2SO_4$: 0,5 (g), KH_2PO_4: 1 (g), glucose: 10 (g), algae powder: 20 (g), and distilled water: 1000 (ml). Successful fermentation for membrane production could be done in appropriate pH of 5 and appropriate temperature of 32^0C. The ratio of surface area per volume of fermentation (S/V) is 0.8, and the membrane can be collected after 5 days.

Màng cellulose vi khuẩn (Bacterial cellulose; Biocellulose; BC) do vi khuẩn Gluconacetobacter tạo ra có những đặc tính vượt trội so với cellulose của thực vật về độ dẻo dai, độ bền, chắc khỏe và độ đàn hồi. Đối tượng: vi khuẩn có khả năng tạo màng Bacterial cellulose từ môi trường tảo xoắn Spirulina. Mục tiêu: Khảo sát ảnh hưởng của một số yếu tố đến quá trình tạo màng Bacterial cellulose trên môi trường tảo xoắn Spirulina, từ đó tìm ra được môi trường dinh dưỡng và điều kiện thích hợp cho quá trình lên men tạo màng Bacterial cellulose. Kết quả: tuyển chọn được 2 chủng vi khuẩn Gluconacetobacter xylinus T_6 và Gluconacetobacter xylinus T_9 có khả năng tạo màng cellulose có đặc tính mỏng, nhẵn, đồng đều, dai phù hợp với các tiêu chí làm mặt nạ dưỡng da. Xác định được môi trường thích hợp cho sự hình thành màng Bacterial cellulose gồm $(NH4)_2SO_4$: 0,5 (g), KH_2PO_4: 1 (g), glucose: 10 (g), bột tảo: 20 (g), nước cất 1000 (ml) với thời gian thu màng là 5 ngày, pH thích hợp là 5 và nhiệt độ thuận lợi cho quá trình lên men tạo màng là 32^0C, tỉ lệ diện tích bề mặt trên thể tích lên men là S/V = 0,8.

Keywords: Gluconacetobacter, Bacterial cellulose, Spirulina

1. Introduction

Bacterial cellulose (or Biocellulose; BC) membrane formed by the bacterium *Gluconacetobacter,* has greatly similar structures and characteristics to cellulose in plants as they both consist of glucose molecules linked together by a β-1,4glucosidic bond [5]. Unlike plant cellulose, Bacterial cellulose does not contain such compounds as ligin, pectin, hemicellulose and wax, etc. As a result, Bacterial cellulose membrane has superior properties forits toughness, durability and elasticity. Thanks to its unique elasticity, Bacterial cellulose membrane is thought to be a new sourse of polymer and a solution to the search for new materials these days.

Raw materials used to cultivate *Gluconacetobacter* are available and diverse: old coconut water, molasses, cane water and rice water, for example. In this study, we choose

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to use *Spirulina*. If used frequently, *Spriulina* can help improve mental strength, fitness and beauty for users of different ages and genders. Thus, there is little doubt that *Spirulina* is an ideal medium for the culture of bacteria *Gluconacetobacter.* From these reasons mentioned above, we decided to do a research on the influences of some ecological factors on the Bacterial cellulose (BC) membrane forming process in *Spirulina* medium in order to find out appropriate and favourable media and conditions for the culture of selected bacteria *Gluconacetobacter.* In combination with the study on harvested membrane treatment process, this study largely contributes to the production of 100% - natural nourishing face masks for users.

2. Materials and research methods

2.1 Stocks

The bacteria seperated from vinegar-fermented membrane samples in *Spirulina* medium.

2.2 Bacterial culture media

Medium for bacteria isolation (MT1) is composed of Glucose: 20 g, $(NH_4)_2SO_4$: 3 g, $MgSO_4.7H_2O$: 2 g, agar - agar: 20 g, peptone: 5 g, KH_2PO_4: 2 g, $CaCO_3$: 10 g, acetic 2 %, distilled water: 1000 ml, pH 5.5.

Medium for membrane-forming fermetation (MT2) is composed of Glucose: 10 g, $(NH_4)_2SO_4$: 0,5 g, $MgSO_4.7H_2O$: 1 g, KH_2PO_4: 1 g, acetic 2 %, *Spirulina* powder: 20 g, distilled water: 1000 ml.

2.3 Methods

***Microbiological methods*:**

Isolating and selecting bacteria strains that are capable of forming Bacterial cellulose membrane: Isolating bacteria by critical dilution method [4].

Dying, observing cells in electronic microscopes: Get colonies in the tilt jelly tube, make smeared on a slide, fixed the smeared by heating a flame, staine cells by Gram stain method. Put on the template with objective 10-40 to observer then move the template under 100 oil objective with a magnification of 1000 times. If bacterial cells change to pink, they will be Gram-negative bacteria. If bacterial cells change to purple, they will be Gram-positive bacteria.

Biochemical methods:

Examining catalase activity: Drop a H_2O_2 3% onto the surface of colonies. In the presence of bubbling, bacterial strains will have catalase activity (catalase +). If not, they haven't catalase activity (catalase -).

Examing the aibility of ethanol oxidation into axetic: Culture bacterial strains which selected on the medium, including (g/l): yeast extract: 10 g, ethanol: 10% -15% (vol/vol), water: 1000 ml, pH 6.8 to 7.0, Blue bromophenol (BPB) 0.04% 20 ml. In the presence of acetic acid, the medium will change from blue to yellow.

Identifying the ability of acetic acid oxidation: Culture bacterial strains which selected on the medium, including (g/l): yeast extract: 10 g, Calcium acetate: 10 g, agar: 20 g, water: 1000 ml, pH 7.0 to 7.2. In the presence of milk while ring, reaction will be positive. If not, reaction will be negative.
Examing the ability to metabolize glycerol into dihydroxyaceton: Culture bacterial strains which selected on the medium, including (g/l): yeast extract: 0.3 %, corn extract: 3 %, Glycerol: 4 %; $CaCO_3$: 0.3 %, $(NH_4)_2SO_4$: 0.1 %, water: 1000 ml, pH: 5.3. Drop Fehling's solution to test the dihydroxyaceton formation (with the appearance of precipitate Cu_2O - red brick).

3. Findings and discussion

3.1. Isolating and selecting possible microorganisms for the production of Bacterial cellulose membranes in *Spirulina* medium

We isolated bacteria in a particular medium with raw Spirulina. 15 pure bacterial strains were collected. Having dyed Gram of these collected bacterial strains, they were all found to catch pink dye. Hence, it can be said that all selected bacterial samples are Gram-negative. Furthermore, check the biochemical characteristics of the bacterium Gluconacetobacter, result as follows:

Table 1. The biochemical characteristics of the bacterium *Gluconacetobacter*

Ordinal	Characteristics	Phenomenon	Result
1	Oxidation of ethanol into axetic	Medium with Bromphenol Blue 0.04% change from blue to yellow	+
2	Catalase activity	The presence of bubbling	+
3	Growth on Hoyer medium	Undeveloping biomass	–
4	Metabolize glycerol into dihydroxyaceton	Appearance of precipitate Cu_2O - red brick after fermentation	+
5	Metabolize glucose into acid	Halo appears around the colonies on medium containing $CaCO_3$	+
6	The ability born brown pigment	No brown pigmentation	–
7	The ability to synthesize cellulose	The scum of bacteria appear blue	+

Thus, six samples of bacteria called T_4, T_6, T_8, T_8, T_{10}, T_{12} were premilinarily selected. They belong to *Gluconacetobacter xylinus* species, *Acetobacteriaceae* family, *Gluconacetobacter* genus.

Six selected strains of bacteria are then cultured in humoral environment at the temperature of 30°C to observe the formation of *Bacterial cellulose* membrane. The results revealed that 2 strains of *Gluconacetobacter* bacteria prove able to produce cellulose membrane characterized by its thinness, smoothness, toughness and unifomity, matching the criteria of nourishing face masks production. Having compared these two bacterial strains, we found that the bacteria T_9 can produce thinner, smoother and stronger membrane in an earlier time, so we decided to choose the bacteria T_9 as the subject of further study.

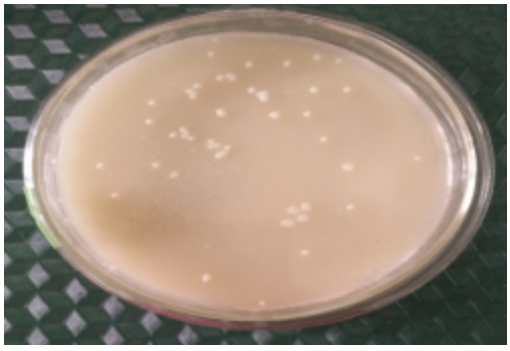

Figure 1. Colonies of isolated bacteria

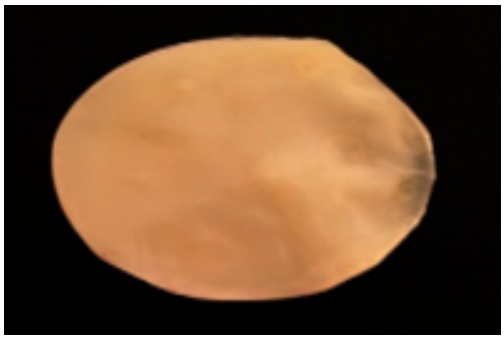

Figure 2. BC membrane produced from bacteria T9

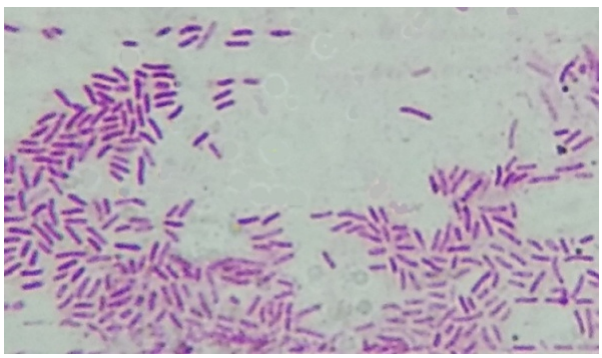

Figure 3. Cytological morphology of bacteria T_6 (X 1000)

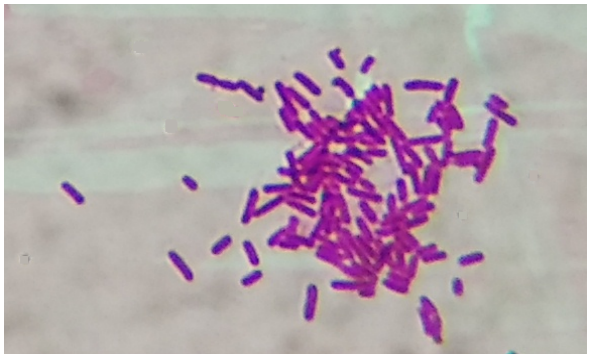

Figure 4. Cytological morphology of bacteria T_9 (X 1000)

Bacteria T_9 are bacilli (rod-shaped bacteria), being individual or arranged in sequence. They are also characterized by their immobility, catalase activity and the oxidation of etylic alcohol into axetic acid. They can metabolize glycerol into dihydroxyaceton or glucose into gluconic acid. They do not grow in Hoyer medium [3].

We selected 2 out of 15 strains of *Gluconacetobacter* bacteria that could produce Bacterial cellulose mambrane use in nourishing face masks production for its thinness, smoothness, toughness and uniformity.

3.2. Determing the appropriate concentration of *Spirulina* for the formation of Bacterial cellulose membrane

In order to determine the appropriate amount of *Spirulina* for the Bacterial cellulose membrane formation, we changed the concentration of *Spirulina* from 0.1 to 30 grams per litre. Results are shown in the following chart:

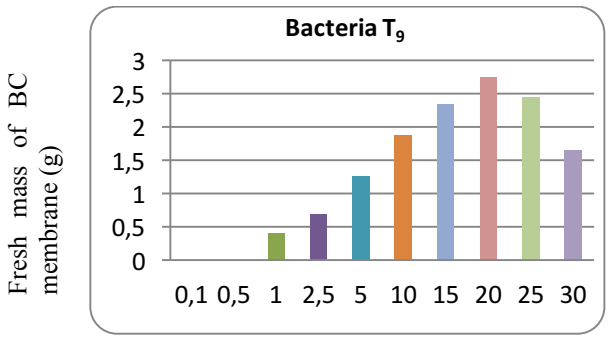

Figure 5. Influence of spirulina concentration on fresh mass of BC membrane

As can be seen from the chart, the thickest Bacterial cellulose membrane is produced at the *Spirulina* concentration of 20 g/l. With less than 15 g/l *Spirulina*, the fresh weight of Bacterial cellulose membrane is smaller due to the lack of nutrients for the growth of bacteria. However, the growth of bacteria is also restricted if the concentration of nutrients is more than 20 g/l.

Thus, it can be concluded that 20 g/l is the most suitable Spirulina concentration for the Bacterial cellulose membrane formation.

3.3. Influence of carbon on the formation of Bacterial cellulose membrane

In order to studythe influence of carbon on Bacterial cellulose membrane formation, we first used *glucose, saccharose, ethanol* as the main sources of carbon. After 4 days, Bacterial cellulose membranes were collected to examine the influence of carbon sources on their formation. We found that glucose helps produce the membrane with the highest weight, so we decided to choose *glucose* as the carbon source of research. The amount of glucose varies from 0 to 20 g/l, and the findings are shown in the chart below:

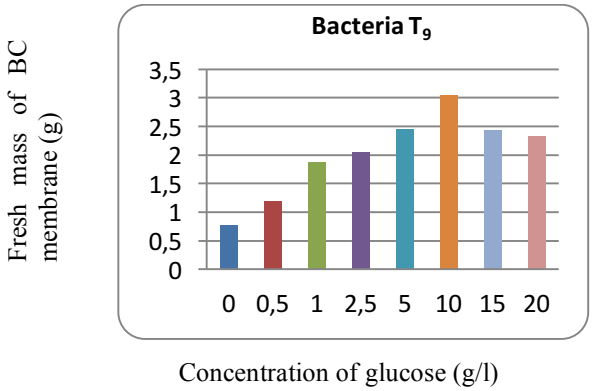

Figure 6. Influence of glucose concentration on fresh mass of BC membrane

As illustrated from the chart, the thickest Bacterial cellulose membrane is produced at the glucose concentration of 10 g/l. With less than 5 g/l, less glucose is produced due to the insufficient amount of cutured humour for the growth of bacteria. As a result, the Bacterial cellulose membrane weighs less. In contrast, when the concentration of glucose is more than 15 g/l, it cannot be used up by bacteria. Excess glucose converts into gluconic acid, reducing pH and thus inhibiting the synthesis of glucose.

To sum up, 10 g/l is the most appropriate amount of glucose for the Bacterial cellulose membrane formation.

3.4. Examing the influence of $(NH_4)_2SO_4$

NH_3 and NH_4^+ are considered the most easily-absobed source of nitrogen for microorganisms. Hence, $(NH_4)_2SO_4$ is selected as the nitrogen source of research. The concentration of $(NH_4)_2SO_4$ varies from 0 to 3 g/l, and the findings are illustrated in the chart below:

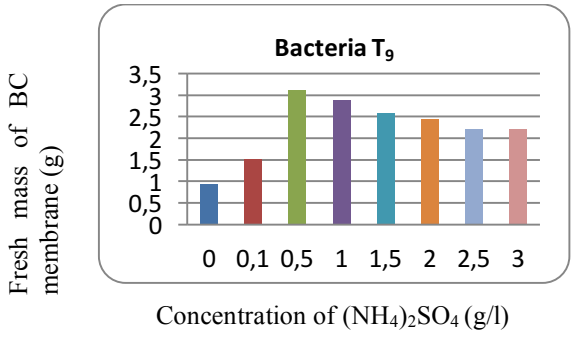

Figure 7. Influence of $(NH_4)_2SO_4$ concentration on the fresh mass of BC membrane

As can be seen from the chart, with the $(NH_4)_2SO_4$ concentration of 0.5 g/l, the thickest Bacterial cellulose membrane is produced. At the amount of more than 1 g/l, the fresh mass of Bacterial cellulose membrane is small. It can be explained by the fact that when the bacteria do not use up the nitrogen, excess nitrogen inhibits the growth of bacteria.

Hence, 0.5 g/l is the most appropriate concentration of $(NH_4)_2SO_4$ for Bacterial cellulose membrane formation.

3.5. Examining the influence of KH_2PO_4

The presence of phosphorus compounds and its concentration in the solution has a strong influence on the metabolism of microorganisms. This leads to our decision of studying the influence of KH_2PO_4 on the formation of Bacterial cellulose membrane by bacteria T_9. The concentration of KH_2PO_4 varies from 0 to 3 g/l. The resuts are shown in the following chart:

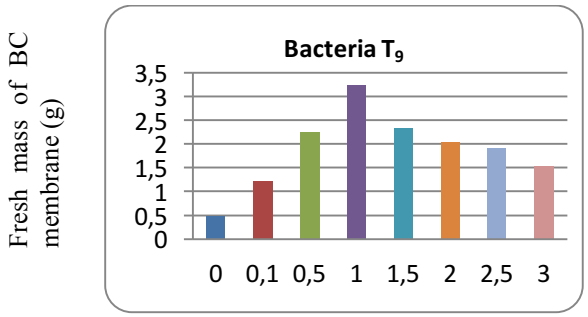

Figure 8. Influence of KH_2PO_4 on the fresh mass of BC membrane

As illustrated in the chart, bacterium T_9 produces the thickest Bacterial cellulose membrane at the KH_2PO_4 concentration of 1 g/l. When KH_2PO_4 concentration is less than 0.5 g/l, the fresh mass of Bacterial cellulose membrane is small as too low concentration of phosphorus and potassium affects their participation in the formation of coenzyme catalysts for reactions in the growth process. On the other hand, more than 1.5 g/l KH_2PO_4 leads to the excess of phosphorus and potassium, changing the physical and chemical characteristics of the environment and inhibiting the growth of bacteria.

To conclude, 1 g/l is the most appropriate concentration of KH_2PO_4 for the formation of Bacterial cellulose membrane.

3.6. Influence of culture time on the formation of Bacterial cellulose membrane

When culturing *Gluconacetobacter xylinus* T_9 in the medium MT2 at the temperature of 30^0C, we found out that: On the first day, due to the movement from breeding environment into the fermentation one, there is a sudden change in environmental conditions. As a result, the number of cells is not large enough to produce cellulose. After 2 days, secondary threads appear in succession in aligned holes on the surface of bacterial cells. Then they twist together to form microfibers settling to the bottom. Next, cellulose

strips overlap and twist together to form thin white sheet of cellulose on the surface. On the third day, a very thin cellulose membrane appears on the surface of fermentation vessel. The amount of cellulose booms from the fouth day to the sixth day of culture process. This is the time for the maximum cellulose biosynthesis of bacteria thanks to environment adaptation and sufficent cells.

Table 2. Influence of culture time on the formation of BC membrance from bacteria G. xylinus T_9

Ordinal	Days	Properties of Bacterial cellulose membrane	M ± m	δ (%)
1	3	Very thin, transparent and smooth	1.17 ± 0.02	0.03
2	4	Thin, transparent and smooth	1.25 ± 0.01	0.02
3	**5**	**Thin, transparent, tough and smooth**	**2.45 ± 0.02**	**0.03**
4	6	Holes on the surface	3.05 ± 0.01	0.02
5	7	Suspended in the solution	3.65 ± 0.02	0.03
6	8	Near the bottom of fermentation vessel	4.05 ± 0.01	0.02

M: Average mass of the membrane
m: Mean error
δ: Average squared deviation

As can be seen in Table 2, the fresh mass of *Gluconacetobacter xylinus* T_9 membrane increases with time of culture. The longer culture time is, the greater Bacterial cellulose membrane is. The volume of membrane increases sharply from Day 4 to Day 6, from 1.25 ± 0.01(g) to 3.05 ± 0.01(g) On the 6^{th} day, the growth of membrane decreases constantly. The difference between Day 6 and Day 7 is about 0.60 (g) compared to 1.20 (g) between Day 4 and Day 5. There is a slight increase between Day 7 and Day 8. As the research aims at thin and tough Bacterial cellulose membrane production, we choose to collect the membrane on Day 5.

To conclude, thin and tough Bacterial cellulose membrane is best produced by culturing Gluconacetobacter xylinus T_9 in the time length of 5 days.

3.7. Influence of pH on the formation of Bacterial cellulose membrane

pH is measured by the concentration of ions H^+ and OH^- in the solution. pH of the solutionhas direct and indirect influence on the cells' physiological properties, it also affects the possibility of forming Bacterial cellulose membrane of bacteria *Gluconacetobacter xylinus* T_9 [2].

To find down the appropriate pH for the growth of cells in Bacterial cellulose membrane, we culture the bacteria in some media with different pH values at the same temperature of 30^0C in 5 days. The results are illustrated as follows:

Table 3. Influence of pH on the formation of BC membrane from bacteria G. xylinus T_9

Ordinal	pH	Propeties of Bacterial cellulose membrane	M ± m	δ (%)
1	3	Thin, transparent, smooth	1.27 ± 0.02	0.03
2	4	Thin, tough, smooth	2.59 ± 0.01	0.02
3	**5**	**Thin, transparent, tough, smooth**	**3.15 ± 0.02**	**0.03**
4	6	Thin, tough, viscous	2.62 ± 0.01	0.02
5	7	No formation		
6	8	No formation		

M: average mass of membrane
m: Mean error
δ: average squared deviations

Table 3 shows that different amounts of cellulose are collected with the use of different pH values of solution. Along with cellulose synthesis of *Gluconacetobacter* bacteria is its cellulase synthesis. The more cellulase is produced, the less possibility of polimerization bacteria haveto produce cellulose. Thus, less cellulose is produced. With high pH (pH > 5), more cellulase is produced than cellulose, and vice verse, cellulose production increases if pH is less than 5. In addition, cellulose synthesis and glucose oxidation give more ions for the metabolism, which reduces the amount of glucose used for the cellulose formation. The appearance of byproducts when glucose is oxidised by some enzymes could be used to explain why different species of microorganisms perform different cellulose biosynthesis at different pH values and with different sources of carbon [1]. *Gluconacetobacter xylinus* T_9 bacteria are cultured in *Spirulina* medium in 5 days at pH 7. At pH 8, there is no Bacterial cellulose membrane formation. From pH 4 to pH 6, membranes with the similar thickness are formed. At pH 5, the mass of cellulose reaches the highest; tough and smooth Bacterial cellulose membranes appear. These results are in light of those in the study by Hestrin and

Schramm (1954) [6]. pH 5 is determined the optimum to minimize the change of ion concentration in culture environment, so it is selected to culture bacteria T_9.

In conclusion, pH 5 is the most appropriate for the fermentation in Bacterial cellulose membrane formation of bacterium G. xylinus T_9.

3.8. Influence of temperature on the formation of Bacterial cellulose membrane

Not only does temperature affect the growth but it also playsadecisive role in the formation of Bacterial cellulose of bacteria G. xylinus T_9. When being cultured, bacteria grow slowly at low temperatures, so the time of culture is longer. This reduces the possibility of cellulose synthesis. High temperatures can lead to strong respiration, negatively affecting cellulose synthesis [7]. In order to determine the best temperature for fermentation, bacteria G. xylinus T_9 are cultured in warm Binder container (Germany) at pH: 5, but at different temperatures. After 5 days, Bacterial cellulose membranes are collected to determine their fresh weight. Results are shown in the following table:

Table 4. Influence of temperature on the BC membrane formation from bacterium G. xylinus T_9

Ordinal	Temperature (^{0}C)	Prperties of Bacterial cellulose membrane	M ± m	δ (%)
1	15	No formation		
2	20	Thin cellulose thread ssuspended in the solution		
3	**25**	**Thin, transparent, tough, smooth**	**2.85 ± 0.01**	**0.02**
4	28	**Thin, tough, smooth**	**3.00 ± 0.01**	**0.02**
5	30	**Thin, tough, smooth**	**3.25 ± 0.02**	**0.03**
6	32	**Thin, tough, smooth**	**3.34 ±0.02**	**0.03**
7	35	**Thin, tough, smooth**	**3.12 ± 0.01**	**0.02**
8	40	No formation		
9	45	No formation		

M: average mass of membrane
m: Mean error
δ: average squared deviation

As can be seen from Table 4, temperature has a great effect on bacteria *G. xylinus* T_9 like other species. A factor that decides the growth of bacteria is the susceptibility to temperature in reactions catalyzed by enzymes. At low temperatures (15^0C-20^0C), enzyme-catalyzed reactions occur slowly, restricting the growth of bacteria. This only produces cellulose threads suspended in the solution and no formation of membrane appears. Like other chemical reactions, the rate of enzyme-catalyze dreactions increases when the temperature increases. This is followed by the increase of metabolism and quick growth of bacteria. Thus, more cellulose is produced (25^0C: 2,85 ± 0,01 g; 30^0C: 3,25 ± 0,02 g; 35^0C: 3,12 ± 0,01 g). However, at a certain level, bacteria grow slowly although temperature still increases. It is because the activation of enzyme system changes by heat. (40^0C-45^0C: no cellulosemembrane formation) [6]. Each spieces of micoroorganism has its own temperature limit for its growth. 25- 35^0C is the best temperature for the growth and cellulose synthesis of bacteria *G. xylinus* T_9. Bacterial cellulose membrane is best produced at the temperature of 32^0C. Temperatures of over 35^0C can inhibit the cellulose synthesis.

In general, the most appropriate temperature for the Bacterial cellulose membrane formation from bacteria G. xylinus T_9 is 32^0C.

3.9. Influence of surface area and fermentation volume (S/V) on the formation of Bacterial cellulose membrane

Having examined the formation of Bacterial cellulose membrane of bacteria *G. xylinus* T_9 with different ratios S/V, results are shown in Table 5.

Bacteria *G. xylinus* are obigateearobes, so oxygen is the essential element for their growth. Accordingly, the ratio S/V has a great influence on the formation of Bacterial cellulose membrane. Data in Table 5 show that Bacterial cellulose membrane is best formed when the ratio S/V is 0.8 and the depth of solution is 1.20 cm.

Table 5. Influence of surface area and fermentation volume on the BC membrane formation from bacterium G. xylinus T_9

Ratio S/V (cm^{-1})	h (cm)	Properties of Bacterial cellulose membrane	M ± m	δ (%)
0.5	2.20	Very thin, amorphous	0.65 ± 0.02	0.03
0.6	1.70		1.05 ± 0.02	0.03

Ratio S/V (cm^{-1})	h (cm)	Properties of Bacterial cellulose membrane	M ± m	δ (%)
0.7	1.45	Poorly amorphous	2.00 ± 0.01	0.02
0.8	**1.20**	Smooth, tough, 1.5-3 mm thick	**3.33 ± 0.02**	**0.03**
0.9	1.10		3.17 ± 0.01	0.02
1.0	1.00		3.05 ± 0.01	0.02
1.5	0.60	Very thin, torn	1.12 ± 0.01	0.02

M*: average mass of membrane*
m: Mean error
δ: average squared deviation
S: surface area of culture (cm^2)
V: fermentation volume (cm^3)
h: Depth of fermentation solution (cm)

4. Conclusion

In the study, 6 strains of *Gluconacetobacter xylinus* bacteria were isolated, but we found that T_6 và T_9 have the best formation of Bacterial cellulose membrane, which matches the criteria of the nourishing face masks production. The ideal nutrional medium for the formation of Bacterial cellulose membrane in *Spirulina* from *G. xylinus* T_9 is composed of: (NH4)2SO4: 0.5 (g), KH_2PO_4: 1 (g), glucose: 10 (g), alega powder: 20 (g), distilled water: 1000 (ml). The time of bacteria culture is 5 days, appopriate pH is 5, appropriate temperature is 32^0C, and the ratio between surface area and solution volume (S/V) is 0.8.

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