

Production of Biopolymer from *Acetobacter xylinum* Using Different Fermentation Methods

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Abstract- Researchers all over the world have been studying microbial cellulose - which is a biopolymer - for more than a century as it is receiving great attention for its wide applications. Many researches have been carrying out in order to find better methods of microbial production at a large scale. Using Rotary Discs Reactor (RDR) is one of the alternatives to produce higher yield of microbial cellulose. This study has been done to compare production of microbial cellulose using RDR with the traditional static fermentation method. A series of fermentation in static culture and RDR were run at room temperature with pH ranging from 4 to 6. Results showed that fermentation carried out using RDR gave 86.78% higher production of microbial cellulose than that from static fermentation. In this study, RDR fermentation with speed rotation of 7 rpm gave the highest resultant wet weight which was 139.78 grams. It was also found that too high rotational speed in RDR caused decrease in microbial cellulose production as it affects stability of the culture. It can be concluded that fermentation using RDR can give higher yield because of better oxygen supply where the *A. xylinum* cultures were in direct contact with air by the rotation of discs. Furthermore, the RDR provided larger surface area for the microorganisms attachment compared to the static fermentation.

Keywords: Microbial cellulose, rotary disc reactor, static fermentation, dissolved oxygen, *A. xylinum*

I. INTRODUCTION

The American Chemical Society reported in February 2007 in the Science Daily [1] that biotechnology's next high-value product could be microbial cellulose, a form of cellulose produced naturally by bacteria. It is believed that this unique cellulose has made a comeback. The distinctive properties of microbial

cellulose make it suitable to be used in different fields such as bioprocessing, medical, food and paper manufacturing [2], [3], [4]. Moreover, microbial cellulose has finer structure compared to plant cellulose and does not contain hemicelluloses or lignin that need to be removed prior to processing. Furthermore, it can be grown to almost any shape. Industrially, microbial cellulose was produced for products such as dessert, wound dressing, high strength paper and diet foods.

Microbial cellulose is produced from bacteria coming

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from many species; however, only the *Acetobacter* species produce enough cellulose to justify commercial interest. The most extensively studied member of the *Acetobacter* species is *Acetobacter xylinus*, formerly known as *A.xylinum* which was used in this study as cellulose producer. It is gram negative bacteria that can be found naturally in ripened and rotten fruits where the inert surroundings of these bacteria make it very sensitive to harsh environment. Reference [5] reported that harsh environment did not affect *A.xylinum* growth but indirectly affect cellulose production since cellulose negative mutant of *A.xylinum* will be produced. The production of microbial cellulose by *Acetobacter xylinum* can be grown under a variety of conditions which are static cultures, submerged cultures and rotating disc bioreactor [6]. Each method has its own unique properties as well as advantages and disadvantages.

Static fermentation is simple and does not need high technology but there are many disadvantages such as diffusion problem, difficulties in monitoring the fermentation condition and scale up. Scaling up of the production is laborious, tedious and uneconomical for commercialization. Many of the limitations in static cultures can be overcome in agitated culture by using Continuous Stirrer Tank Reactor (CSTR). The product from the agitated medium is not in pellicle form but rather is formed as reticulated cellulose slurry. This technique also comes with culture instability problems demonstrated by loss of the ability to make cellulose and the gradual overgrowth of cellulose producing cells by cellulose non producers (Cel⁻) types. Interestingly, in RDR, discs alternately soak the organisms in the medium and expose them to air providing better aeration compared to conventional method. Apart from that, the discs

provide larger surface area for cellulose attachment to both sides of the discs hence give RDR many advantages which are higher production yield, less labour and ease for up scaling.

This study was done to compare microbial cellulose production in static fermentation and in Rotary Disc Reactor. Current experimental procedures run the rotating disk bioreactors in batch mode.

II. MATERIALS AND METHODOLOGY

A. Preparation of Medium

Starter medium for *Acetobacter xylinum*

100ml of Shigeru Yamanaka medium was prepared per 1 litre of distilled water containing 50g sucrose (QRec), 5g yeast extract (Bacto), 5g ammonium sulphate (QReC), 3g potassium dihydrogen phosphate (QReC) and 0.05g magnesium sulphate (QReC). The medium was stirred until all ingredients dissolved. The pH of the medium was adjusted with acetic acid to pH 5.0 by using pH meter. Then the medium was poured into a flask, closed with cotton, covered with aluminium foil and then put to autoclave at 121°C for 15min. After cooled down to room temperature, 10ml of *Acetobacter xylinum* (MARDI, Serdang) were added to the medium using aseptic technique. The solution was mixed apparently by shaking the flask slowly. Next, the flask was closed with cotton, covered with aluminium foil and placed in an incubator (Mettler 400, Germany) for 4 days at 28 °C.

Fermentation medium

About 1 litre of Shigeru Yamanaka medium was prepared. The medium was stirred until all ingredients dissolved. The pH of medium was adjusted with acetic acid to pH 5.0 by using pH meter

then transferred to 1litre schott bottle for autoclave at 121°C for 15min. After being cooled down to room temperature, 100ml of inoculums was added to the medium with aseptic technique. The solution was mixed apparently by shaking the schott bottle slowly.

B. Experimental Setup

Rotary disc reactor (RDR)

The RDR that we developed in this research was constructed using Poly(methyl methacrylate) (PMMA) with a stainless steel shaft. The discs used were made of stainless steel with mesh sizes of 0.3cm. About 39% of the discs was immersed in the medium and exposed to air when it rotated. Disc dimension was 7 cm in diameter and 1 mm thick, which gave surface area of 77cm² for each disc. However, the overall active surface area for each disc was 71.9cm² as only 39% of the disc directly contacted the medium. Eight discs were mounted on a horizontal steel shaft which gives 575.2cm² of total surface area. The shaft will rotate with various type of speed from 7 – 11 rpm using an 18W driven electronic motor. Total volume of designed RDR was 2L with 1L of working volume. Medium with inoculums was poured into RDR. The RDR was covered with aluminium foil and left for five days fermentation at room temperature with a rotating speed of 7 rpm and pH 4. After microbial cellulose was harvested, the experiment was repeated with different rotation speed range from 7 – 14 rpm and pH 4-6 at room temperature to determine the best condition for RDR operation.

Static fermentation

For static fermentation, the shaft with discs was taken out from RDR. The trough now will act as a tray to run static fermentation. This provides 200cm² active surface areas for fermentation, which is 2.5 times

lower compared to total active surface area for RDR. Medium with inoculums was poured into a tray that had similar calculated surface area as RDR. The tray was covered with aluminium foil and left for five days static fermentation at room temperature. After 5 days, microbial cellulose was harvested and the experiment was repeated with different pH range from 4 to 6 to compare the production with RDR.

C. Data Analysis:

Measurement of wet weight and dry weight of microbial cellulose

Microbial cellulose from fermentation was harvested and rinsed with distilled water. Then the cellulose was washed with NaOH solution 2M. After that the cellulose was rinsed again with plenty of distilled water and dried with tissues. The clean microbial cellulose was weighed using Electronic Balance (DENVER, Germany) to get the wet weight of microbial cellulose. The microbial cellulose then was put in an oven for 1 day at 60°C to remove water content and gain the dry weight.

Dissolve Oxygen Measurement

A dissolved oxygen (DO) probe, Oxy-Check (Hanna Instrument, Romania) was used to measure the oxygen in static culture and the rotating disc reactor. The probe was calibrated in aerated tap water to get the saturated (100%) signal. The probe was placed directly in the RDR and was cleaned routinely to prevent fouling of the tip. Data were stored as percent saturation, but could easily be converted to mg/l by changing the measurement in the instrument.

Statistical Analysis

Statistical analysis was performed using analysis of variance (ANOVA) Two Factor without replication in Microsoft Word Excel 2007 at $p > 0.05$.

III. RESULTS AND DISCUSSION

A. Microbial cellulose production yield after five days fermentation

Figure 1 show microbial cellulose produced in RDR and in static fermentation. For five days fermentation at fixed condition which is pH5 and room temperature, RDR produced 139.78 gram wet cellulose while static culture gave only 17.88 gram. This shows that RDR achieved 86.78% more yield of cellulose than that from static fermentation. For dried weight, RDR gave 80.77% higher yield compared to static fermentation. Result of the experiment shows that fermentation of RDR produced microbial higher water content compared to static fermentation. Microbial cellulose produced by RDR fermentation gave 96.13% water content while for static fermentation it was 94.18%. This agrees with [7] that reported microbial cellulose produced by using rotating discs concept contains higher water compared to static.

See Fig 1

From statistical analysis, ANOVA Two Factor without replication had been run in order to prove the ability of RDR to increase microbial cellulose production statistically. From ANOVA that had been run, it gave p -value equal to 0.0183 which is smaller than 0.05. The result also shows that F ratio value of the test, 52.894 is larger compared to F critical value, 18.512. The F-test output proved that the difference between RDR and static fermentation is statistically significant. Therefore, the significant capability of RDR to produce higher yield of microbial cellulose was statistically proven.

One of the reasons behind this significant increment is aeration factor. Previous studies reported that static culture had a diffusion limitation problems. Microbial cellulose film that produced at the surface of medium will slowly restrict the diffusion of oxygen and nutrient. This will lead to inactivation or death of *A.xylinum* because of lack of oxygen and food. In RDR, the discs rotate and let *A.xylinum* in contact with the medium and air during fermentation. This automatically reduces or perhaps eliminates the diffusion limitation problems as in static culture fermentation.

Another factor that lead to increase of microbial cellulose production in RDR is the larger surface area. The cellulose production was found to be proportional to the surface area of the culture when volume were held constant [8]. In RDR, the surface area for cellulose to attach on are higher compared to static fermentation. In this study, with good discs packing, active surface area of RDR can form up to 2.5 times compared to static fermentation. Therefore, it is demonstrated that fermentation using RDR give better cellulose yield compared to static fermentation. These phenomena are due to the fact that RDR gives better aeration for microbial cellulose production and at the same time provides larger surface area for liquid-air interface.

Effect of pH in Static and RDR Fermentation

A series of experiments of static and RDR fermentation with different initial pH had been run in order to investigate the effect of production methods to pH drops. Fermentation conditions were fixed at room temperature for 5 days and 7rpm (for RDR).

The conversion of glucose to gluconic acid resulted in significant drop in pH of the medium in batch culture

[9]. Figure 2 shows initial and final pH for static and RDR fermentation. Both methods show that the value of pH drops is proportional to pH increase.

See Fig 2

However, the value of pH drops for RDR fermentation was much higher compared to static fermentation. Static fermentation gave value of pH drops in the range of 0.07 to 0.2 while for RDR fermentation was from 0.21 to 0.93. This result was expected as limited oxygen in static fermentation will also inhibit gluconic acid production.

Figure 3 shows microbial cellulose production in the RDR and static fermentation at different pH. It shows that pH 5 allows the highest cellulose weight production for RDR which is 139.78 g while that at pH 4 for static fermentation. This finding is supported by previous discussion of pH drops. It shows that pH for both fermentation throughout the process agrees with the optimum pH for cellulose development as reported by [6] that rapid cellulose production occurs between pH of 3.5 to 7 with the highest rate of formation at around pH 4.5 to 6. These data agree with previous investigations [8], [10] and there seems to be a consensus regarding the optimum pH range for cellulose production [11], [6].

See Fig 3

B. Comparison between Static and RDR fermentation

It was clearly observed that fermentation using RDR gives consistently significant increase in microbial cellulose yield compared to static fermentation. From the experiments, two major factors that lead to increase of microbial cellulose weight in RDR were

identified. The first is surface area for fermentation while the other one is aeration factors. Microbial cellulose yield is proportional to surface area for fermentation [8]. It was mentioned that RDR with good discs packing provides better surface area compared to static fermentation which reduces the diffusion problem as in static fermentation. Figure 4 visually illustrates diffusion problems in static fermentation and how RDR overcomes the problems.

See Fig 4

It describes that the cellulose layer formed at the interface of static fermentation causes limitation for food and oxygen diffusion. In RDR, microbial cellulose formed at the surface of the rotating discs which allow *A.xylinum* to be exposed to air for oxygen and to medium alternately. This technically reduced diffusion problem in static fermentation. However, the speed of rotation should be controlled to achieve optimum microbial cellulose production. Microbial cellulose production decreases with increase of rpm even though dissolved oxygen increases. This is due to harsh environment and excessive oxygen resulted from high rotating speed. Hence, it is crucial to achieve both optimum rotation speed and medium conditions for the fermentation.

IV. CONCLUSION

Static culture fermentation for microbial cellulose production was compared with that in rotary disc reactor (RDR). At similar fixed conditions, it was shown that fermentation using RDR gives better cellulose yield compared to static method. This result is expected due to better aeration and higher surface area provided in the RDR where more than 80%

increment in cellulose wet weight production was achieved in comparison to static fermentation. Consequently, dissolved oxygen was analysed throughout the fermentation process to study the effect of rotational speed towards dissolved oxygen concentration. It was found that dissolved oxygen (DO) concentrations increase with the increase of rotational speed. In the contrary, too high rotational speed resulted in decrease of microbial cellulose production since it affects the stability of the culture. From that, it can be concluded that a critical limit of dissolved oxygen exists whereby below that gives significant effect to microbial cellulose yield in the RDR. It shows that in RDR, *A.xylinum* are in direct contact with the air by rotation of the discs and do not depend solely on dissolved oxygen in the medium.

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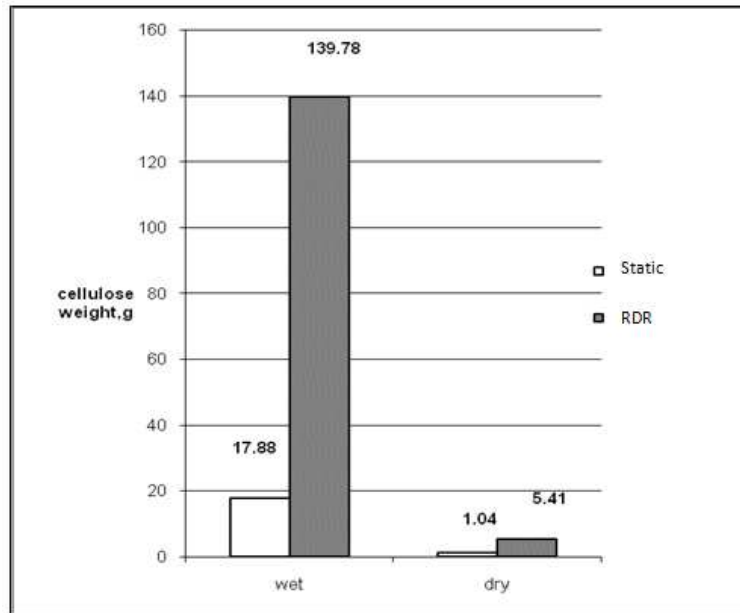


Fig 1: Cellulose produced between static and RDR fermentation after 5 days.

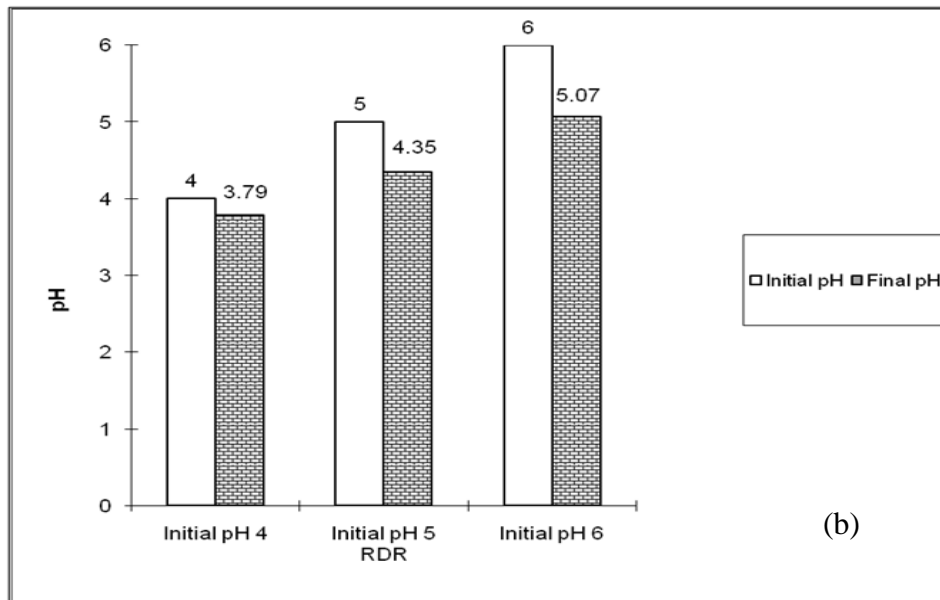
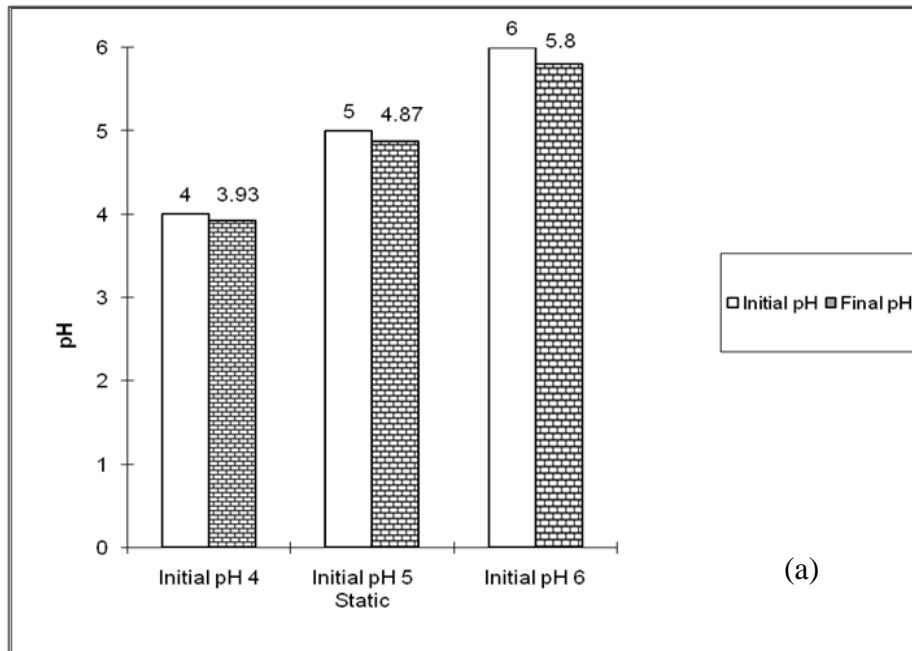


Fig 2: Value of pH drop in (a) static and (b) RDR fermentation after 5 days.

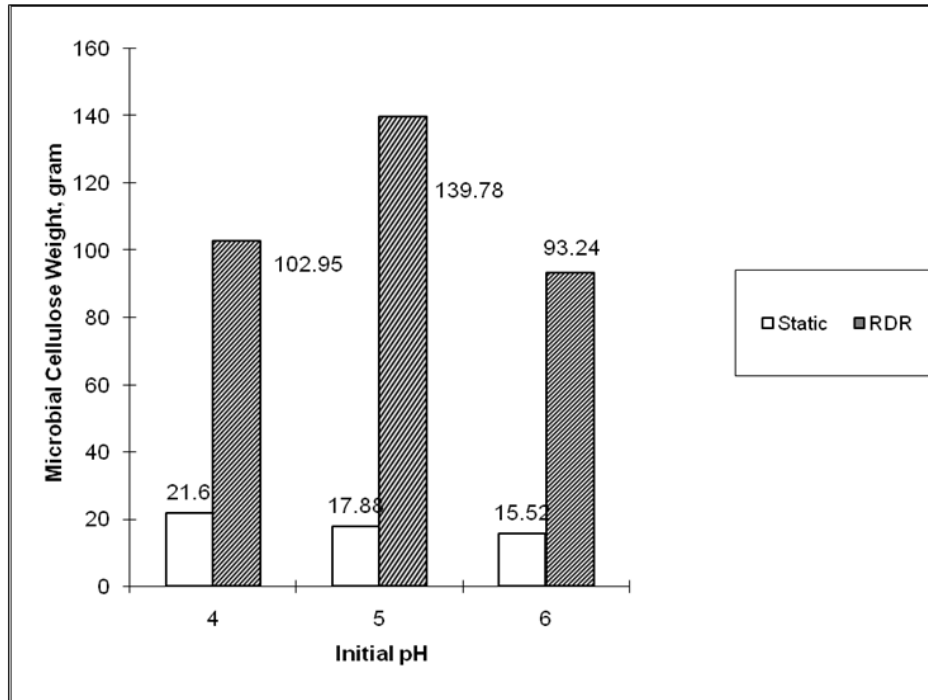


Fig 3: Comparison of cellulose wet weight using static and RDR fermentation at different initial pH

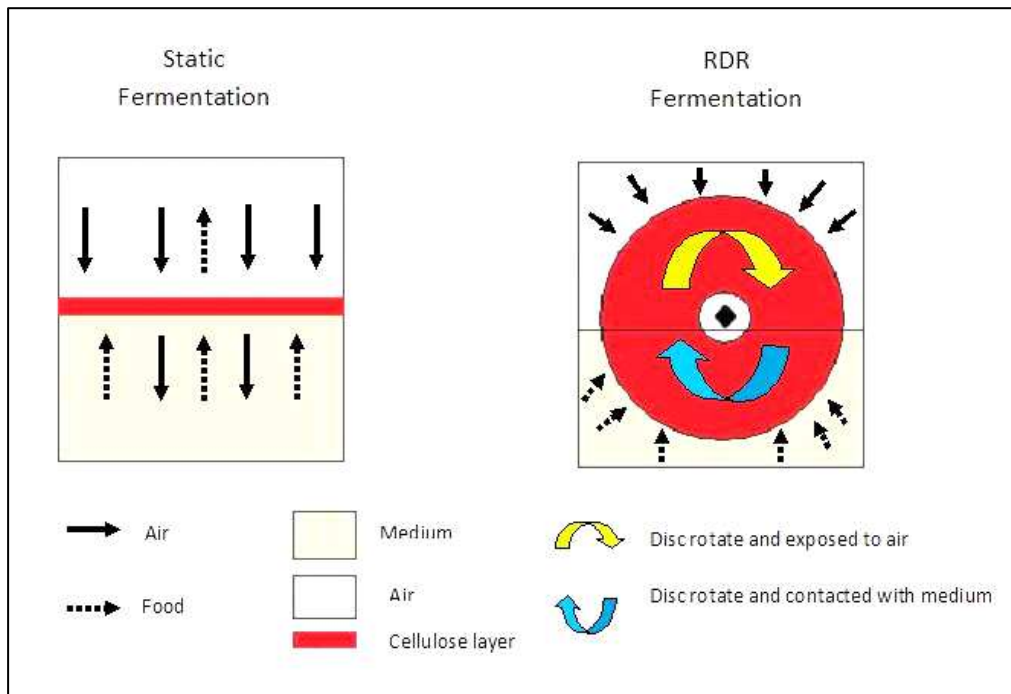


Fig 4: Comparison of air and food diffusion in static and RDR fermentation