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Research Article

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Optimization of the Production of Microbial Cellulose by *Acetobacter xylinum* in *Aloe barbadensis* Mill. Medium

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ABSTRACT

Aloe barbadensis Mill. was used as an alternative medium for growing A. xylinum culture to produce Microbial Cellulose (MC). A one year and a six months old A. barbadensis Mill. leafs were mixed to produce A. barbadensis Mill.-medium (ABM-medium). The mixture ratio of the one year and six months old Aloe barbadensis Mill. are 100%:0%; 75%:25%; 50%:50%; 25%:75%; 0%:100% respectively. Distilled water was used as control. These media were then fermented for seven days in 28 ^{0}C. The dry and wet weight data of the produced MC were collected and analyzed using one way-ANOVA. The result showed that ABM-media produced more MC than the control ($P \leq 0,05$) and the optimum ratio of the ABM-medium is 0% old:100% young based on the wet and dry weight. This result was then used in the second experiment. Initial pH of 3, 5, 7 and 9 were combined with glucose concentration of 0% (control); 2,5%; 5%; 7,5%; 10%; 12,5% and 15% using factorial ANOVA design to get the optimum combination of both in the ABM-medium. After seven days of fermentation in 28 ^{0}C

the result showed that the optimum combination of all is pH 3 with 15% glucose ($P \leq 0.05$) with the highest wet and dry weight of MC.

***Keywords**: Acetobacter xylinum, Microbial cellulose, Aloe barbadensis Mill., ratio, medium.*

INTRODUCTION

Microbial Cellulose (MC) is one of the polysaccharides produced by microbes such as *Acetobacter xylinum*[2]. This complex compound has been used in the food industry to produce nata de coco[3] and nata de pina[14]. In the last few years MC has been used in burn injury treatment[4]. To refine the usage of MC in this treatment, an alternative medium with capability against contaminant is needed. *Aloe barbadensis* Mill. is used in this research to make *A. barbadensis* Mill.-medium (ABM-medium). It has supportive properties, which help in the curing process of burn wounds[16] and also has anti-bacterial, anti-virus and anti-fungal agents, which are able to prevent contamination in the growth medium[13].

MATERIALS AND METHODS

Acetobacter xylinum culture is retrieved from the collection of Microbiology Laboratory of University of Western Australia. *Aloe barbadensis* Mill. leafs are retrieved from P.T. Alove Bali Indonesia, Br. Tengah-Bonbiyu, Ds. Saba, Blahbatuh, Gianyar – Bali. The *Aloe barbadensis* Mill. leafs taken are one year of age (labeled as old) and six months of age (labeled as young). The leafs are taken from the outer most part of the plant.

Hestrin and Schramm (HS) broth and agar is used as medium, which contain 2 gram glucosa, 0,5 gram yeast extract; 0,5 gram pepton; 0,27 gram sodium phosphate; 0,12 gram citric acid and diluted in distilled water and the end volume was made to 100 mL[5]. For making the agar HS medium, 2 gram agar is added to the medium with the same ingredient.

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This medium and the equipment are then sterilized using autoclave at the temperature of 121 ^{0}C and the pressure of 15 lbs for 15 minutes. The medium and the equipment are stored in the fridge and in the oven respectively until needed in this research.

***Acetobacter xylinum* starter preparation**

The starter is made by inoculating the *A. xylinum* stock culture to the slanted medium prepared before and incubated in 48 hours at room temperature. This young culture is than inoculated to the HS broth medium and incubated in 48 hours at room temperature and used as an active starter on MC production.

***Aloe barbadensis* Mill. Gel extraction**

A. barbadensis Mill. leaf washed by water before and cleaned by using 70% alcohol, is peeled by using a knife to get the gel. The retrieved gel is filtered with a sterile filter cloth and accommodated in to a beaker glass.

Growth curve

To know the timing of inoculation, a growth curve is needed. For this purpose a calculation of total colony numbers using pour plate plating method is to be done.

Determination of the optimum ratio of the young and old *A. barbadensis* Mill. mixture in ABM-medium.

This experiment is done in a beaker glass with a 100 mL capacity. The old and young *A. barbadensis* Mill. gel are mixed together to produce *A. barbadensis* Mill.-medium (ABM-medium). The mixture ratio of the one year and six months old *Aloe barbadensis* Mill. are 100%:0%; 75%:25%; 50%:50%; 25%:75%; 0%:100% respectively. Each ratio combination are placed in to a beaker glass and each of them is mixed with 0,5 g glucose[7] and 0,15 mL glacial acetic acid[15]. And then each beaker glass is inoculated with 0,5 mL *A. xylinum* starter with the most optimum age as an inoculate (obtained from the growth curve result). The end solution volume is made into 50 mL. Beaker glass filled with only distilled water has a role as a control. All of the solution are incubated for 168 hours (seven days)[8]. The wet and the dry weight of the harvested MC are to be determined. Each treatment is repeated four times and the result are averaged.

The examination of optimum glucose concentration and optimum pH for *A. xylinum* in producing MC in ABM-medium.

The impact of glucose addition, medium start pH and the interaction between these two factors will be taken in account in this experiment using the ABM-medium with the optimum ratio, obtained from the result of previous experiment. In this experiment completely randomized factorial design was being used, which combined two independent variables (glucose concentration and start pH). The glucose concentration in the medium is adjusted to (w/v) 0% (as control); 2,5%; 5%; 7,5%; 10%; 12,5% and 15%. The pH medium is adjusted to 3, 5, 7 and 9.

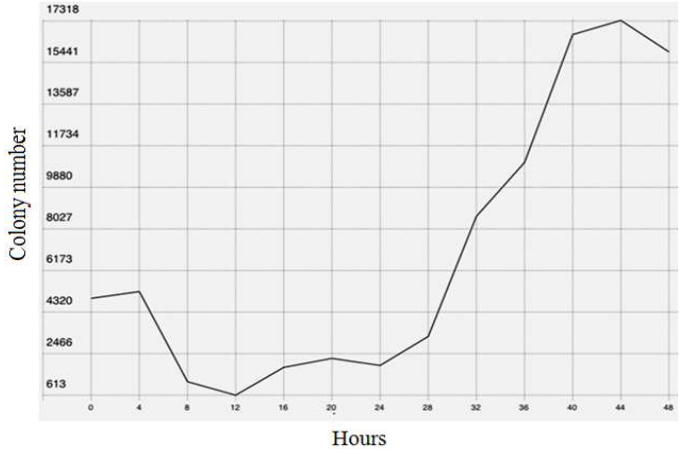
All of the medium is inoculated with 0,5 mL *A. xylinum* starter with the most optimum age as an inoculate (obtained from the growth curve result) and incubated for 168 hours (7 days). The wet and the dry weight of the harvested MC are to be determined. Each treatment is repeated four times and the result are averaged. The datas obtained from the experiment are analized using ANOVA with SPSS (version 17.0). If the obtained results have a significant difference ($P \leq 0,05$) this test will be continued with Duncan test.

RESULTS AND DISCUSSION

The Growth Curve of *A. xylinum*

The result of the growth curve of *A. xylinum* beeing presented on Graph 1 shows that the lag phase is located between 8 and 28 hours. In this phase a decrease of cell numbers occures. Probably it is caused by a large number of dead cells as a result of the differences of the condition between the previous and the new medium so that an adaptation time is needed to synthasize enzymes and the component, which is important for *A. xylinum* growth.

Graph 1: Growth curve of *A. xylinum*



Log phase is located between 28 and 40 hours. Willey *at al*. (2008) explained that at this phase, bacterial cells grow and divide at the maximal rate and the growth rate is constant[18]. The bacterial population condition in this phase mostly uniform, chemically and physiologically. The growth rate in this phase is also constant, so that it is appropriate to be used in the next experiment. From the log phase, the 36th hour is taken as the optimum inoculation time.

Stationary phase is located between 40 and 44 hours. Willey *at al*. (2008) explained that at this phase the bacterial cell number remain constant[18]. This is because between the dividing cell and the dead cell is in balance or the bacterial cell does not divide anymore but still doing its metabolic process. The bacteria enter this phase for several reasons, first because of the nutrient limitation in the medium and the second is because of the metabolic waste accumulation, which is very toxic, so that the bacterial growth becomes slow. After this phase comes the death phase started at 44 hours and on. In this phase the nutrition has depleted severely and the concentration of toxic metabolic waste has become very high which is causing the death of the most bacterial cell.

Determination of the optimum ratio of the young and old *A. barbadensis* Mill. mixture in the ABM-medium.

The result was measured on the 7th day. The observed parameters were wet weight (WW) and dry weight (DW) of the yielded MC in gram.

Table 1 ABM-medium mixture ratio

No	Treatment	$\bar{x}$ WW (g)	$\bar{x}$ DW (g)
1	Control	0,33±0.05$^{(a)}$	0,01±0.01$^{(a)}$
2	100% Old : 0% Young	0,83±0.10$^{(b)}$	0,04±0.00$^{(b)}$
3	75% Old : 25% Young	0,86±0.07$^{(b)}$	0,05±0.00$^{(c)}$
4	50% Old : 50% Young	0,82±0.16$^{(b)}$	0,05±0.00$^{(b)}$
5	25% Old : 75% Young	0,96±0.09$^{(b)}$	0,0525±0.01$^{(c)}$
6	0% Old : 100% Young	1,13±0.13$^{(c)}$	0,0500±0.00$^{(c)}$

Values in Tabel 1 ± standard deviation are means of four times repetition. Numbers followed by the same letters show no significant different based on Duncan test with the significant level of 0,05 using SPSS software for windows ver. 17.0.

The control has a lower wet and dry weight ($P \leq 0.05$) in compare to the treatment. The treatment of 0% Old : 100% Young shows the highest wet weight in compare to the other treatment ($P \leq 0,05$). The highest dry weight is produced by the treatment of 75% Old : 25% Young, 25% Old : 75% Young and 0% Old : 100% Young, wherein these three treatments have no significant different ($P \geq 0,05$). All of these points are summarized in Table 1.

Based on the measurement of WW and DW, the treatment of 0% Old : 100% Young is chosen to be the optimum ratio, because this treatment has the highest WW and DW in compare to the other treatment, moreover the structure and form of the MC yielded by this treatment either in WW or in DW are the best in compare to the other treatment.

There is a contamination of *Aspergilus niger* in the control, on the other hand there is not a single fungi contamination found in the ABM – medium. This indicates that the ABM-medium has an anti-fungal agent.

The 0% Old : 100% Young ratio based on the above analysis is the most optimum ratio of yielding MC. It is probably because the young *A. barbadensis* Mill. gel has more nutrition than old *A. barbadensis* Mill.. As has been mentioned by Ray *at al*. (2013) that minerals such as calcium (Ca), potassium (K), magnesium (Mg) and phosphorus (P) are more on the young *A. barbadensis* Mill. than the old one[10].

Minerals such as Ca, Mg, and P play an important role in producing MC. As has been explained by Ross *et al*. (1987), that Ca regulates PDE A, namely in c-di-GMP degradation process[11]. The c-di-GMP self has a role in activating cellulose synthase. This means that Ca indirectly has an important role in regulating MC production. As well as Ca, Mg also has a significant role in regulating cellulose synthase. Mg is a cofactor on a conversion reaction of 2GTP into c-di-GMP by diguanylate cyclase. Mg also has a key role on PDE A and B in inhibiting c-di-GMP, by degrading it into 2 GMP molecules. P has a notable role in *A. xylinum* metabolic reaction, which is integrated in nucleotide combination such as ATP, GTP, c-di-GMP and so on. It has been explained that GTP, which than be converted to c-di-GMP is the main activator of cellulose synthase. The existence of P becomes very crucial, since this element is a part of chemical compound, which activates the MC production.

Based on field observation, old *A. barbadensis* Mill. gel is more viscous than the young *A. barbadensis* Mill. gel. Based on the statement of Saibuatong and Phisalaphong (2009) this high viscosity can lower the oxygen transfer rate in the medium[12]. *A. xylinum* is an aerobic bacteria, which needs oxygen in its metabolic processes, so that the limited oxygen transfer rate will interrupt the metabolic process of this bacteria, and one of it is the MC production.

The examination of optimum glucose concentration and optimum pH for *A. xylinum* in producing MC in ABM-medium.

The observed results from this experiment are the WW, DW in gram and the end pH after seven days of fermentation.

Table 2 The average MC WW in gram after seven days of fermentation

Glucose concentration (%)	pH			
	3	5	7	9
0	1.61±0.08$^{(g)}$	0.00±0.00$^{(a)}$	0.00±0.00$^{(a)}$	0.00±0.00$^{(a)}$
2,5	2.34±0.28$^{(h)}$	0.65±0.22$^{(def)}$	0.69±0.18$^{(ef)}$	0.29±0.41$^{(abcde)}$
5	2.34±0.80$^{(h)}$	0.60±0.11$^{(def)}$	0.44±0.12$^{(bcdef)}$	0.18±0.24$^{(abc)}$
7,5	2.67±0.19$^{(h)}$	0.59±0.09$^{(def)}$	0.54±0.08$^{(cdef)}$	0.19±0.39$^{(abc)}$
10	3.42±0.16$^{(ij)}$	0.78±0.07$^{(f)}$	0.57±0.14$^{(cdef)}$	0.00±0.00$^{(a)}$
12,5	3.29±0.19$^{(i)}$	0.62±0.10$^{(def)}$	0.45±0.09$^{(bcdef)}$	0.07±0.14$^{(ab)}$
15	3.75±0.22$^{(j)}$	0.67±0.19$^{(def)}$	0.45±0.15$^{(bcdef)}$	0.27±0.39$^{(abcd)}$

Values in Tabel 2 ± standard deviation are means of four times repetition. Numbers followed by the same letters show no significant different based on Duncan test with the significant level of 0,05 using SPSS software for windows ver. 17.0.

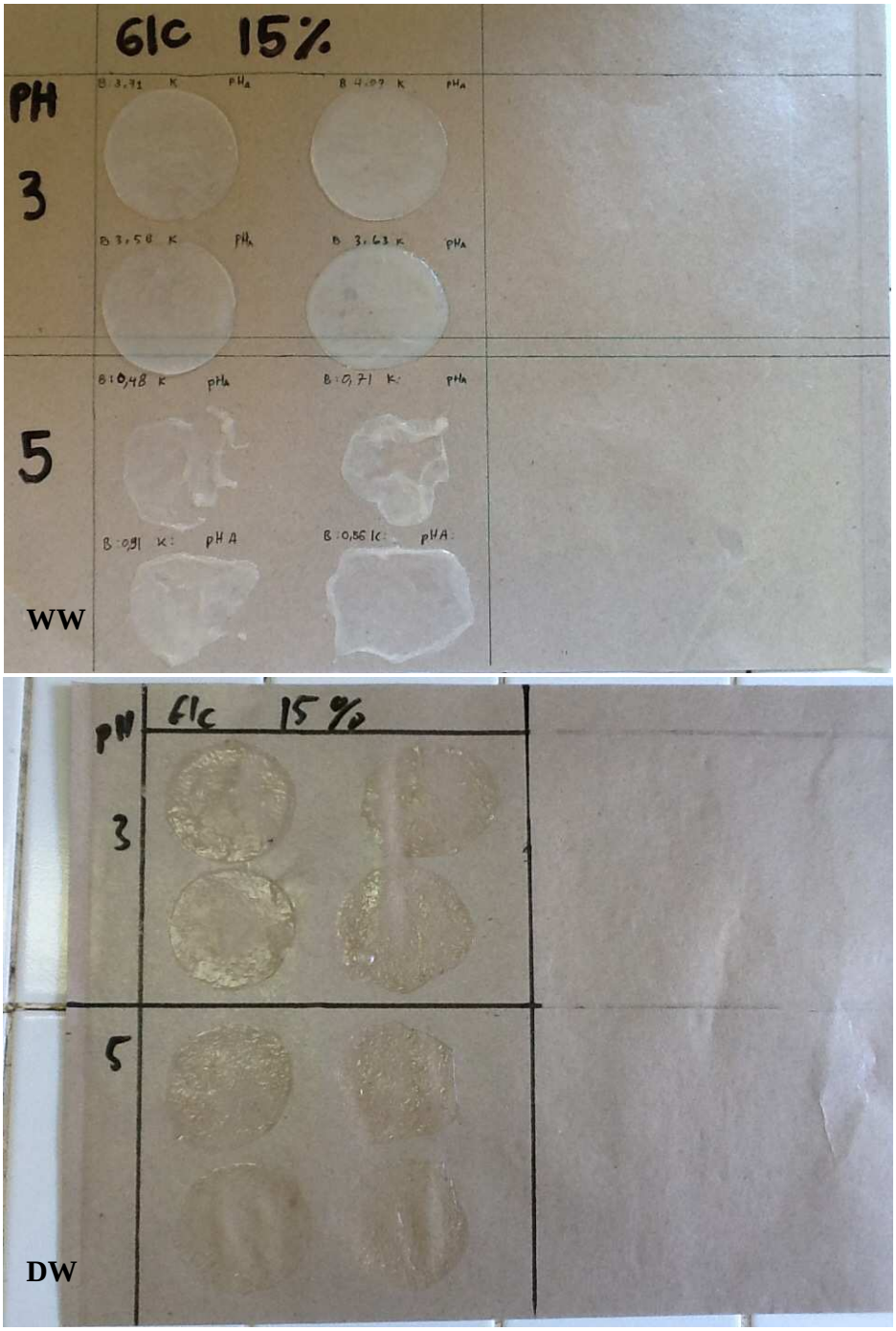
Table 3 The average MC DW in gram after seven days of fermentation

Glucose concentration (%)	pH			
	3	5	7	9
0	0.05± 0.01$^{(abcde)}$	0.00±0.00$^{(a)}$	0.00±0.00$^{(a)}$	0.00±0.00$^{(a)}$
2,5	0.14± 0.01$^{(h)}$	0.05±0.01$^{(bcde)}$	0.04±0.01$^{(abcde)}$	0.01±0.02$^{(ab)}$
5	0.19±0.06$^{(i)}$	0.06±0.01$^{(cde)}$	0.04±0.03$^{(abcde)}$	0.02±0.02$^{(abc)}$
7,5	0.30±0.02$^{(j)}$	0.07±0.02$^{(ef)}$	0.07±0.01$^{(e)}$	0.02±0.04$^{(abcd)}$
10	0.45±0.03$^{(k)}$	0.11±0.03$^{(gh)}$	0.08±0.02$^{(efg)}$	0.00±0.00$^{(a)}$
12,5	0.55±0.02$^{(l)}$	0.11±0.03$^{(fgh)}$	0.06±0.02$^{(de)}$	0.01±0.03$^{(ab)}$
15	0.72±0.05$^{(m)}$	0.13±0.04$^{(h)}$	0.08±0.02$^{(efg)}$	0.05±0.06$^{(bcde)}$

Values in Tabel 3 ± standard deviation are means of four times repetition. Numbers followed by the same letters show no significant different based on Duncan test with the significant level of 0,05 using SPSS software for windows ver. 17.0.

Table 3 and 4 show that the best combination between pH and glucose concentration is pH 3 with 15% or 10% glucose for WW, and pH 3 with 15% glucose for DW. The result show that pH 3 produce MC with the highest WW and DW ($P \leq 0,05$) in compare to all pH. And also the glucose concentration of 15% and 10% in WW data, produce the highest MC in that pH, and both of them are not significantly different ($P \geq 0,05$). However these two glucose concentrations are significantly different in DW data ($P \leq 0,05$), namely the 15% glucose concentration yields more DW of MC than 10% glucose concentration. So that the concentration which produce the highest WW and DW is 15%. Moreover the MC consistency in DW with 15% glucose is sturdier than MC yielded by 10% glucose (Fig.1). Thus it can be concluded that pH 3 with 15% glucose concentration is the most optimum combination.

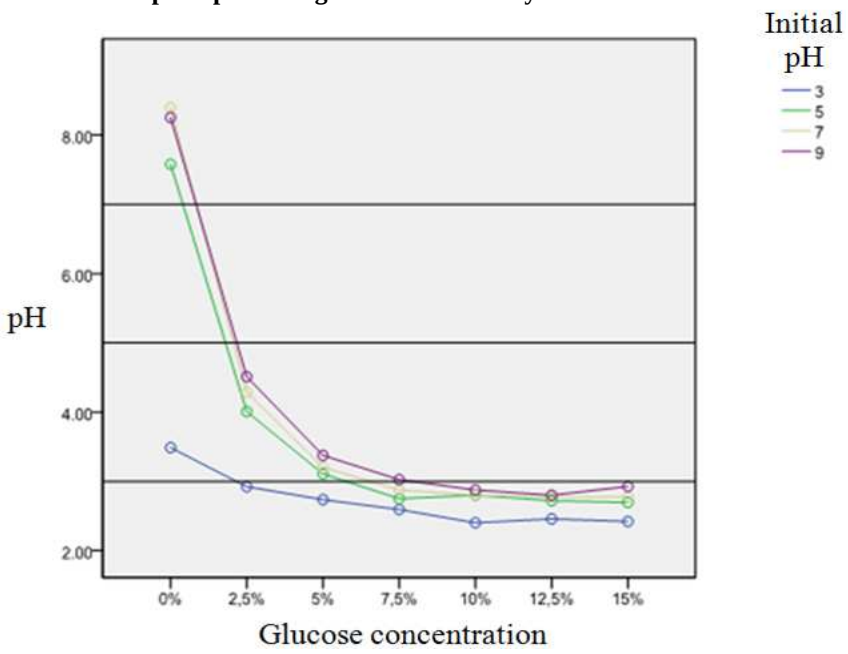
Fig.1:MC produced in pH 3 and 5 with 15% glucose concentration in four replications WW (Wet Weight); DW (Dry Weight)



The Changes of pH After Seven Days of Fermentation

The low production of MC by the *A. xylinum* in pH 5, 7 and 9 raises the researcher interest to know and to explain the cause of it. After seven days, end pH is described in a graph to see the changes from the initial pH. This data is presented on Graph 4. All initial pH has changed. On the glucose concentration of 0%, the initial pH 3, 5 and 7 raised to a baser pH. pH 3 raised to ± pH 3,5; pH 5 raised to ±7,6 and pH 7 became ± 8,4. Whereas initial pH 9 decreased to ± 8,3. Furthermore between 0% to 2,5% concentration, a sharp decline is observed in pH 5, 7 and 9 ($P \leq 0,05$). Even though a decline is also observed in pH 3, the slope is not as sharp as the other three pH, however the decline is still significant ($P \leq 0,05$).

Graph 2. pH Changes After Seven Days of Fermentation



Starting from 2,5% toward 7,5% glucose concentration, a significant decrease is observed on initial pH 5, 7 and 9 ($P \leq 0,05$), however pH 3 is still decreasing until it reaches 10% glucose concentration ($P \leq 0,05$). The decline of all of these pH is heading to one direction, namely between pH 3 and 2. Starting from 10% until 15% glucose concentration, all initial pH have no more significant changes ($P \geq 0,05$). Graph 4 shows that the *A. xylinum* is trying to adjust the environment pH into the more acidic pH. Probably the optimum pH from the *A. xylinum* used in this experiment is between pH 3 – 2,5. It can be seen from the absence of a significant changes when the pH reaches pH 3 – 2,5.

The low production of MC by the *A. xylinum* could be caused by the following terms. In its metabolism, *A. xylinum* produces Gluconate-6-phosphate, which is an organic acid. This organic acid can lower the pH. According to Weinhouse and Benziman (1973) the Gluconate-6-phosphate derived from Glucose-6-phosphate could not be processed further to the Pentose Phosphate Pathway[17], so that it is accumulating until its concentration becomes very high, which results in a pH decrease. the inhibition of the metabolic process for Gluconate-6-phosphate is possible by raising the ATP concentration produced by tricarboxylic acid cycle. Lessie and Vander Wyk (1972) stated, that the high ATP concentration came from the tricarboxylic acid cycle can inhibit Gluconate-6-phosphate dehydrogenase enzyme[6], so that the Gluconate-6-phosphate can't enter the Pentose Phosphate Pathway. Probably the tricarboxylic acid cycle obtains the metabolite from the anaplerotic reaction. Rassow *et al*. (2006) stated the anaplerotic reaction is all reaction form, which is able to give metabolites from outside to the tricarboxylic acid cycle, for instance through the catabolic process of amino acid, which is transformed into the tricarboxylic acid element[9]. For example amino acids like alanine, cysteine, glycine, serine, threonine and tryptophan can be transformed to oxalocetate through pyruvate. Asparagine and aspartate can be changed directly into oxalocetate.

Aspartate, phenylalanine and tyrosine can be transformed to fumarate, and the other anaplerotic reaction involving the 20 amino acids known. According to Barcroft *and* Myskja (2009) these 20 amino acids are contained in the *A. barbadensis* Mill. gel[1] therefore the tricarboxylic acid can run.

However the accumulation process of Gluconate-6-phosphate probably has an impact on the decline of MC production, because the raw material for producing MC namely Glucose-6-phosphate, is used for producing Gluconate-6-phosphate. This is supported by the data above which indicates that MC production in initial pH 5, 7 and 9 is not as much as in initial pH 3. Although MC is difficult to be produced, this Gluconate-6-phosphate accumulation process still produces energy that is aside from the tricarboxylic acid cycle but also from the oxidation of Glucose-6-phosphate itself, which in turn becomes Gluconate-6-phosphate that produces NADH to ATP through respiration chain in mitochondria[9]. It seems that, there is a shifting of balance in the *A. xylinum* metabolic process in environment with to base pH. In this case the metabolic focus is shifted to lowering the pH instead of forming MC.

CONCLUSION

The optimum ratio of the young and old *A. barbadensis* Mill. mixture in the ABM-medium is 0% Old : 100% Young. The optimum glucose concentration and optimum pH for *A. xylinum* in producing MC in ABM-medium is 15% and pH 3 respectively.

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