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Investigating the Conditions for *Nata-de-Coco* Production by Newly Isolated *Acetobacter* sp.

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Abstract

Nata-de-coco, a bacterial cellulose product, which is usually prepared by *Acetobacter xylinum* grown in mature coconut water, is a quite popular snack in Vietnam and other countries. This research was carried out to obtain pure *Acetobacter* strains that can produce high cellulose and to investigate the effect of fermenting conditions on cellulose production. Total aerobic microorganisms in four *nata* fermenting media, collected from local producers in Ben Tre and Vinh Long provinces, Vietnam, were in the range of 8.79-9.14 log cells/mL. Eight strains of *Acetobacter* sp. were isolated and identified by several biochemical and physiological tests. *Acetobacter* sp. strain N was selected as the best cellulose producing bacterium. Mature coconut water that incubated 60 h before fermentation and initial bacterial density of inoculum at 6 log cells/mL gave the highest yield (213.87 g cellulose/300 mL fermented mature coconut water). The addition of glucose into the medium up to 5°Brix was also increased the cellulose production (233.15 g/300 mL). Furthermore, the addition of both (NH₄)₂HPO₄ and (NH₄)₂SO₄ or only (NH₄)₂HPO₄ gave higher cellulose yields, 248.23 and 247.70 g cellulose were obtained from 300 mL mature coconut water, respectively.

1. Introduction

Nata-de-coco is a chewy, jelly-like food, and quite popular snack in Vietnam and other countries. *Nata-de-coco*, a bacterial cellulose product, which is usually prepared by *Acetobacter xylinum* grown in mature coconut water [1, 2]. This product is not high nutrient, less energy and high cellulose content. Therefore, it is useful for decreasing obese people ratio, and stimulating peristaltic intestinal tract that help to regulate excretion easily. This kind of bacterial cellulose (BC) has introduced into many different fields such as paper production, bio-membrane used for treatment of burn and skin trauma and so on. In food, BC was used as membrane for fresh coconut preservation instead of chemical or sausage preservation besides *nata-de-coco* manufacturing. Although there are so many researches on BC now, BC will be still subject that many scientists are interested in the future because of new their applications [3, 4].

Nowadays, the problem of Vietnam and other countries which cultivate and produce products from coconut is the elimination of coconut water is into environment, especially from local producers of coconut's flesh. This is one of causes of environmental pollution. Consequently, using of coconut water for *nata-de-coco* production is necessary because

this work not only reduce the coconut water eliminated into environment but also increase the value of coconut. Ben Tre is known like country of coconut and has many local producers of *nata-de-coco* in Vietnam. However, almost these local producers produce *nata-de-coco* at farmer household scale. Their production process is essential base on their experience and different bacterial sources even reuse bacterium liquid after fermented many times. Hence, quality of *nata-de-coco* is unequal, not stable and low productivity. Many recent researches provided that using pure bacterial starter and ratio of ingredients in producing fermented products and naturally in *nata-de-coco* production kept important roles to effect on productivity and quality of products [5, 6].

Because of important roles and improving ways in the future of BC, this research was carried out to isolate and select good *Acetobacter* strain with high cellulose production and to investigate suitable conditions for effective cellulose production on selected strain.

2. Materials and Methods

2.1 Materials and Chemicals

Nata fermenting media were collected from 4 local producers in Ben Tre and Vinh Long provinces, Vietnam. Microbiological media and chemicals were purchased from commercial products of Hi-Media (India) and Merck (Germany) such as Plate Count Agar (PCA), D-glucose, (NH₄)₂SO₄ (SA), (NH₄)₂HPO₄ (DAP), acetic acid, etc.

2.2. Determination of Total Aerobic Microorganisms

Total aerobic microorganisms in *nata* fermenting media were conducted at 3 continuous diluted concentrations on PCA medium in triplicate and incubated at 30°C. After 24 h and 48 h of incubation, all plates were counted the colonies that were in the range of 25-250.

2.3. Isolation and Identification of *Acetobacter* Strains

Streaked a small amount of *nata* fermenting medium onto mature coconut water medium which consisted of glucose (5 g/L), SA (8 g/L), DAP (2 g/L), agar (40 g/L), and adjusted to pH 4.5 by acetic acid [7]. Coconut water was incubated at 30°C in 24 h and sub-cultured several times until get the purity colonies. Recorded the colony and cell morphology, Gram reaction, catalase test, acetic acid formation on YPDG medium (g/L; D-glucose 5, yeast extract 5, glycerol 5, agar 20) supplemented CaCO₃ (5 g/L) and ethanol (4% v/v), the color changing of YPGD plus 0.02% bromocresol green.

2.4. Screening the Cellulose Production of Isolated *Acetobacter* Strains

Bacterial strains that were preliminarily identified belong

to *Acetobacter* genus were shaking inoculation with the same initial density. After 24 h, determined bacterial density of these strains. Then, these strains were inoculated with the same bacterial densitiy into culture medium. Culture medium using 300 mL mature coconut water, 24-h pre-incubation, was adjusted to 5°Brix. The vessel's size was 17.3 x 13.0 x 7.3 cm. All treatments were conducted in triplicate.

The data were analyzed by Microsoft Excel (version 2007) and Statgraphics Centurion XV (version 15.1.02). Statgraphics Centurion XV was used to test for the least significant difference (LSD) with the confidence interval of 95%.

2.5. Study on Effect of Incubation Time of Mature Coconut Water

Mature coconut water was incubated at environmental temperature (ca. 28-32°C) for 24 h, followed by adjusted total sugar concentration to 5°Brix, pH 4.5 and inoculated with the same initial density at 24, 36, 48, 60 and 72 h in triplicate. Medium used mature coconut water without pre-incubation was as control medium.

2.6. Study on the Influence of Bacterial Cell Density

This experiment was performed to obtain the appropriate bacterial density for highest cellulose production. Initial Brix degree of culture medium was regulated to 5° Brix. The experiment was randomly carried out with 1 factor and 5 levels (3, 4, 5, 6 and 7 log cells/mL) in triplicate.

2.7. Study on the Effect of Sugar Supplementation

Experiment was carried out to investigate initial glucose concentration for most effective cellulose production. The media were supplemented with D-glucose to 5, 6, 7, 8, 9, 10, and 11°Brix. Control medium was without glucose added. All treatments were conducted in triplicate.

2.8. Study on the Effect of DAP and SA

The purpose of this experiment was to obtain nutrient sources added for highest cellulose production. Ratio of DAP and SA added was 0.2% DAP and 8.0% SA, 0.2% DAP and 0% SA, 0% DAP and 0.8% SA, 0% DAP and 0% SA [8, 9].

3. Results and Discussion

3.1. Evaluation of Total Aerobic Microorganisms

Total aerobic microorganisms in 4 samples were in range 8.79-9.14 log cells/mL (Table 1). Result of Nguyen and Pham revealed that *A. xylinum* density was about 7.51-7.68 log cells/mL after 6-day culture [9]. These samples were achieved higher numbers in because the total aerobic microorganisms include other aerobic microorganisms beside of *A. xylinum*.

Samples	Local producers	Total aerobic microorganisms (cells/mL)*	Strains
A	Tran Phuoc	8.92	A1, A2
В	Mai Thi Phuong	9.14	B1, B2
С	Tran Van Chien	9.05	C1, C2, C3
Ν	Hoang Chi Nhan	8.79	Ν

Table 1. Total aerobic microorganisms in nata fermenting medium.

Note: (*) average values of triplicate.

3.2. Isolation and Identification of *Acetobacter* Strains

Eight bacterial strains were isolated successfully from 4 *nata* fermenting medium samples in Ben Tre and Vinh Long provinces, Vietnam (Table 1). The colonies of all isolated strains were circle, swollen, yellowish (Figure 1a). In addition, colonies of A1, B2, C1 and C3 strain had mucous structure. Microscopic examination confirmed that these

strains were rod shaped (Figure 1b, c). Cells shaped of A2, C2, and N strains were shorter than the others (A1, B1, B2, C1, and C3). Biochemical test showed that the catalase was positive reaction and Gram was negative reaction. They formed clear zones on medium containing CaCO₃ and changed the medium color containing bromocresol green from blue to yellow then to green again. Therefore, these strains belong to *Acetobacter* genus [10-12].



Figure 1. Colonies form (a), cells shape (b) and Gram staining (c) of B1 strain.

3.3. Screen of Cellulose Production of Newly Isolated *Acetobacter* Strains

Eight strains were inoculated at the same initial density of 6 log cells/ml into the mature coconut water and incubated in 24 h. The bacterial density was reached 8.60-8.89 log cells/mL (Figure 2). The density of *Acetobacter* sp. N was reached highest number (8.89 log cells/mL) and different significance with the other strains at 5% of means after 24 h incubation.



Figure 2. Density of bacteria strains after 24 h.

Note: Values on the column was triplicate. Means with different subscripts are statistically different at the 95% confidence level.

Cellulose production by these 8 strains after 6 days of fermentation was shown in Figure 3. Strains N, A1, B2, C1 and C3 produced more than 200 g of cellulose per 300 mL of mature coconut water (205.42, 211.78, 204.55, and 211.61 g, respectively) and their production were not significant difference in total cellulose weights at 95% confidence level.



Figure 3. Cellulose yields produced by 8 bacterial strains.

Note: Values on the column was triplicate. Means with different subscripts are statistically different at the 95% confidence level.

Cellulose that *Acetobacter* sp. N produced more white color and smooth surface than other strains (Figure 4). Moreover, statistical analysis of bacterial density at 95% confidence level after 24 h also indicated that density of

Acetobacter sp. N reached highest and difference significance with the other strains. This increasing speed of density will limit the contamination of other microorganisms, so cellulose production will be more effective. Therefore, *Acetobacter* sp. N produced high and nice cellulose was the selected strain using in further studies.



Figure 4. Cellulose of Acetobacter strain B2 (left), N (center), and C3 (right).

3.4. Effect of the Incubation Time of Mature Coconut Water

Mature coconut water was incubated at room temperature, followed by adjusted to 5°Brix, pH 4.5 and inoculated with 7 log cells/mL at 24, 36, 48, 60 and 72 h. Medium used mature coconut water without incubated was as control medium. The yield of cellulose was showed in Figure 5 was varied from 125.23 to 211.80 g per 300 mL of fermented mature coconut water.



Figure 5. Effect of mature coconut water incubated at times on the production of cellulose.

Note: Values on the column was triplicate. Means with different subscripts are statistically different at the 95% confidence level.

Cellulose was produced more effectively with incubated mature coconut water. The yields of cellulose increased with an increase in incubated time of mature coconut water and produced highest at mature coconut water that incubated at 60 h, then yields of cellulose decrease because the nutrient and glucose concentration decrease in increasing incubated time of mature coconut water.

Sucrose was found in freshly coconut water but only glucose was detected after 3-day storage and glucose was

converted mainly to cellulose [7]. When sucrose was used as carbon source for cellulose production, the system included the hydrolysis of sucrose into glucose and fructose as well as other side reactions besides cellulose forming reaction. Therefore, Brix degree decreased more but cellulose production was not high. Besides, the presence of sucrose in the medium in combination with fructose produced lower amounts of cellulose [3].

The addition of acetic acid to the medium enhanced the cellulose yield from glucose, but decreased the cellulose yields on sucrose [13]. Mature coconut water without incubated medium need more acetic acid to adjust the acidity to pH 4.5, which is the optimum pH value for cellulose production. The more incubated time of mature coconut water was, the more sucrose decreased until only glucose was detected. The pH value also decreased and acetic acid added as little, so the yields of cellulose increased. Consequently, the cellulose yield at incubated time of old coconut water of 24, 36 and 48 h was lower than at 60 h although Brix degree decreased more.

Mature coconut water incubated before fermentation is very necessary in production. Materials can be initiative from many places and acetic acid added into medium was saved. The coconut water will not become yellow when cooking. Therefore, the production of cellulose will be more effective.

3.5. Effect of Bacterial Cell Density on Cellulose Production

This experiment was carried out at 3, 4, 5, 6, 7 log cells/mL of bacterial cell densities. The yields of cellulose increased with an increase in inoculum densities, the cellulose production was 94.87-213.87 g/300 mL (Figure 6). The highest of cellulose yields were at initial inocula of 6 log cells/mL (213.87 g/300 mL) and 7 log cells/mL (202.63 g/300 mL), and there was no significant difference at 95% confidence level between these two inoculum levels. However, among the two inoculums levels, 6 log cells/mL was chosen because of saving inoculum into production as well as easy to reach the density.



Figure 6. Effect of bacterial density on cellulose production.

Note: Values on the column was triplicate. Means with different subscripts are statistically different at the 95% confidence level.

3.6. Effect of Sugar Supplementation on Cellulose Production

Fermentation media were adjusted to 5, 6, 7, 8, 9, 10, and 11°Brix by adding D-glucose. The medium without glucose supplemented was control. The highest of cellulose yields was at 5°Brix, and there was significant difference at 5% confidence level with the others (Figure 7). The yield of cellulose decreased with an increase in initial glucose concentration. The decrease in cellulose yield at high initial glucose concentration could due to some glucose metabolized to gluconic acid, whereas forming cellulose. When initial glucose concentration increased, the pH value in the medium after fermentation decreased lower the range 4.0-6.0 which is the optimum pH range for cellulose production because of accumulation of gluconic acid [14]. However, at

initial 4°Brix and 5°Brix, pH value in the medium increased slowly. The gluconic acid increased and then decreased again. pH value, in common with the changing of gluconic acid, also increased and then to decreased again because glucose not used for cellulose synthesis is metabolized via gluconic acid to other substances [13, 14]. Therefore, at initial Brix degree was 4°Brix and 5°Brix which glucose concentration was not high, accumulation of gluconic acid was metabolized to other substances. Consequently, initial glucose concentration supplemented was very important. If initial glucose concentration supplemented was low, there were not enough substances for production of cellulose. On the contrary, the accumulation of gluconic acid inhibited cellulose production.



Values on the column were triplicate. Means with different subscripts are statistically different at the 95% confidence level.

Figure 7. Effect of initial Brix degree on cellulose production.

Besides, mature coconut water will become yellow if it was boiled in long time. As the results showed in Figure 7, treatment of 5°Brix was the best choice because of not high initial glucose concentration will avoid yellow becoming of mature coconut water and the accumulation of gluconic acid and save glucose supplemented on production.

3.7. Effect of DAP and SA on Cellulose Production

DAP and SA added to supply nitrogen source for cellulose production, whereas glucose was supplied as main carbon source for cellulose production of *Acetobacter*. The addition of nitrogen to the medium before fermentation, cellulose production was higher than that without nitrogen source added, 223.87-248.23 g/300 mL and 104.41 g/300 mL, respectively (Figure 8).



Figure 8. Effect of DAP and SA supplemented on cellulose production.

Note: Values on the column were triplicate. Means with different subscripts are statistically different at the 95% confidence level.

These results indicated that this Acetobacter strain need more nutrient added besides glucose and nutrient within coconut water. The addition of both DAP and SA (248.23 g/300 mL) or only DAP (247.70 g/300 mL) gave highest yields, and there was no significant difference at 95% confidence level between two levels. Thus, the addition of DAP only was enough nitrogen supplemented. This result was also agreed with Nguyen Thi Hien [8]. When medium was not added DAP and SA, Acetobacter used glucose converted to cellulose, so Brix degree decreased. Moreover, the Brix degree changing also showed that cellulose production was more effective if Acetobacter were supplied both nitrogen and phosphate source. In fact, DAP and SA were added to medium with higher ratios of 0.2% DAP and 0,8% SA [9]. In addition, these ratios were different between local producers, although these chemicals must be used carefully because cellulose will be process to food. Hence, the results of studies that only addition of DAP, without SA to medium were very significant in production, so both saving to spend on chemicals and more biological safety.

4. Conclusions

Eight *Acetobacter* strains was isolated and identified from 4 *nata* fermenting medium samples. Colonies of all strains were circle, swollen and yellowish color. Some strains have mucous structure. Cells of all strains were short and long rod shaped. Cellulose production by *Acetobacter* sp. N was sufficient from mature coconut water with initial sugar concentration of 5°Brix, culture inoculum of 6 log cells/mL, and pre-incubated in 60 h (233.15 g/300 mL). The addition of both DAP and SA (248.23 g/300 mL) or only DAP (247.70 g/300 mL) gave higher yields of cellulose production.

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