Arquivos de Ciências do Mar

COCONUT WATER, *Cocos nucifera* LINNAEUS, AN ALTERNATIVE GROWTH MEDIUM FOR *Staphylococcus aureus*

Água de coco, *Cocos nucifera* Linnaeus, um meio alternativo para crescimento de *Staphylococcus aureus*

Norma S. Evangelista-Barreto[1,2], Fátima C.T. de Carvalho[2], Francisca G.R. de Menezes[2], Camila M. Silva[2], J.F. Nunes[3], C.C.M. Salgueiro[3], Regina C. Torres[2], Oscarina V. de Sousa[2], Regine H.S. Fernandes Vieira[2]

ABSTRACT

The coconut, Cocos nucifera *L., is an abundant and inexpensive commodity in Brazil. An excellent source of carbohydrates and mineral salts, coconut water was tested in this study as an alternative growth medium for* Staphylococcus aureus *culture. Lyophilized coconut water was hydrated, prefiltered and filter-sterilized with a 0.22 µm Millipore membrane.* S. aureus *was inoculated in coconut water (M1), coconut water enriched with 1% peptone (M2) and BHI (M3, positive control). After 12 hours of shaking at 140 rpm at 35ºC, bacterial growth was observed. Growth rates were good in M2 and M3, with no statistically significant difference. Thus, coconut water enriched with 1% peptone was shown to be a feasible alternative to commercially available growth media for* Staphylococcus aureus *culture.*

***Keywords*:** *coconut water, growth medium,* Staphylococcus aureus.

RESUMO

Devido à disponibilidade do coco, Cocos nucifera *L., no Estado do Ceará, seu baixo custo e a composição química de sua água, rica em carboidratos e sais minerais, este trabalho foi realizado com o objetivo de se estudar o crescimento microbiano da bactéria* Staphylococcus aureus, *utilizando a água de coco como meio de cultura alternativo. Para a realização do experimento a água de coco liofilizada foi hidratada e após uma pré-filtragem foi esterilizada por filtração em membrana Millipore de 0,22 µm. Em seguida,* S. aureus *foi inoculado em três diferentes meios. O primeiro meio contendo apenas água de coco (M1), o segundo contendo água de coco adicionada de 1% de peptona-Difco (M2) e o terceiro o meio – controle, caldo BHI (M3). Após o cultivo de 12 horas, e agitação de 140 rpm a 35ºC observou-se que o crescimento do microrganismo nos meios M2 e M3 não diferiam entre si, estatisticamente, ambos apresentaram bom crescimento microbiano. Conclui-se que água de coco poder ser utilizada como meio base no crescimento de* Staphylococcus aureus *necessitando apenas de uma fonte de nitrogênio adicional.*

Palavras-chave: *água de coco, meio de crescimento,* Staphylococcus aureus.

[1] Universidade Federal do Recôncavo da Bahia, Cruz das Almas, Bahia.
[2] Instituto de Ciências do Mar, Universidade Federal do Ceará, Fortaleza, Ceará.
[3] Universidade Estadual do Ceará, Fortaleza, Ceará.

INTRODUCTION

Brazil is the world's fourth largest producer of coconuts. In 2005 alone, the country produced over 3 million tons, corresponding to 2 billion nuts (FAO, 2006). The largest producers of Northern and Northeastern Brazil are Bahia (35.2%), Pará (12.2%) and Ceará (11.9%) (Agrianual, 2006).

Coconut water, *Cocos nucifera* L., is a low-calorie, nutrient-rich natural drink much appreciated by Brazilian consumers, especially in coastal regions. Consumption of coconut water has increased over the past years, in part due to increased awareness of its potential for electrolyte replacement in case of dehydration or fatigue (Costa *et al*., 2005).

Depending on the species, microorganisms require certain basic nutrients for their development. Macronutrients are necessary in greater amounts as they constitute the main components of cellular organic compounds (~90%). The remaining 10% are micronutrients, such as potassium, calcium, zinc, iron and manganese (BARBOSA; TORRES, 1998). In the laboratory, the nutritional needs of bacterial cells may be supplied by a range of usually costly synthetic growth media (Tortora *et al.*, 2000).

Coconut water contains most nutrients needed for bacterial growth, although the addition of nitrogen appears to be necessary. It consists of 93% water, 5% sugars (glucose, fructose and sucrose), proteins, mineral salts, neutral fats and water-soluble vitamins, including niacin, biotin, riboflavin, folic acid and ascorbic acid (Arouch &; Vianni, 2002; Costa *et al*., 2006). This explains the increasing research interest in coconut water as a culture medium and cell growth promoter in embryos (PINA et al., 2007), as an oral rehydration solution for the treatment of diarrheal diseases (Fagundes Neto *et al*., 1989), as diluent for goat semen (Oliveira et al., 2009), as maturation medium for canine oocytes (PINA et al., 2007) and as growth medium for bovine embryos (Blume *et al*., 1998).

Thus, in view of its abundance, low cost and suitable chemical composition, coconut water was tested in this study as an alternative growth medium for *Staphylococcus aureus*, a nutritionally demanding species commonly used as a biomarker of hygiene and sanitary conditions in food processing.

MATERIAL AND METHODS

The study was carried out at the Laboratory of Seafood and Environmental Microbiology at the Marine Sciences Institute, Federal University of Ceará (UFC), Fortaleza, Brazil.

Staphylococcus aureus ATCC 25923 was activated in brain-heart infusion broth (BHI, Difco) at 35ºC for 24 hours and stored in BHI agar (Difco) until the moment of testing.

Three growth media were tested:

- M1: coconut water broth
- M2: coconut water broth enriched with 1% peptone (Difco)
- M3: BHI broth (Difco), used as positive control

Coconuts of the green dwarf variety, *Cocos nucifera* L., were obtained from an organic farm certified by the Biodynamic Institute (IBD/Ceará). The coconut water was lyophilized by the Biotechnology Laboratory of the Ceará State University (UECE).

Three culture media were prepared, namely:

M1: Lyophilized coconut water buffered to pH 7.3 and 300 mOsm/kg, hydrated in distilled water, prefiltered, filter-sterilized with a 0.22 µm Millipore membrane and aseptically adjusted to pH 7.0 ± 0.2 using 1M NaOH.

M2: Preparation identical to M1, but enriched with a nitrogen source (1% peptone).

M3: Positive control medium (BHI) prepared according to manufacturer's instructions (Difco) and autoclaved at 121ºC for 15 min.

Culture flasks (1000-mL Erlenmeyer) with *S. aureus* (initially 10^4 CFU.g^{-1}) seeded in 300 mL growth medium (M1, M2 or M3) were shaken at 140 rpm (Lab-Line, Orbit) in a water bath at 35 ± 0.2ºC for 12 hours. Absorbance was registered with a spectrophotometer (Micronal, model B542) at 600nm with intervals of 0.007. The inoculum (10^7 UFC.g^{-1}) was obtained after incubation overnight in BHI broth at 35 ± 1.0ºC. All assays were performed in triplicate.

Growth was determined from spectrophotometric absorbance at 600 nm and from plate count (pour plate, PCA, 35ºC ± 1.0ºC for 24-48 hours) using 0.1% peptone water as diluent. Samples (1.5 mL) were collected for spectrophotometry immediately after inoculation (t_0) and at 1-hour intervals for 12 hours. Plate counts were determined using 1-mL aliquots. All assays were performed in triplicate.

Specific growth rates (µ/h) were calculated for *S. aureus* based on growth curves expressed in CFU/mL versus time (h). The specific growth rate corresponded to the angular coefficient (b) of the linear equation ($\hat{Y} = a + bX$) of Log CFU/mL as a

function of time during the exponential growth phase, determined by linear regression.

Nitrogen concentration and substratum type were submitted to bivariate Analysis of Variance, randomized blocks (growth media) were submitted to univariate analysis, and average **b** coefficients were compared with the ***t*** test ($p<0.05$).

RESULTS AND DISCUSSION

Figure 1 shows the growth curves for *S. aureus*. CFU/mL values were transformed into logarithms (log CFU/mL) in order to adjust the data to the linear regression model. Bacterial growth (Table I) was strongest in M3 (positive control) with an increase of 3 log cycles, followed by M2 with 2 log cycles. Growth was negligible in M1. Although coconut water is rich in reducing and non-reducing sugars, minerals, amino acids and certain vitamins (Table II), the addition of nitrogen appears to be indispensable for bacterial growth (Smith & Bull, 1976).

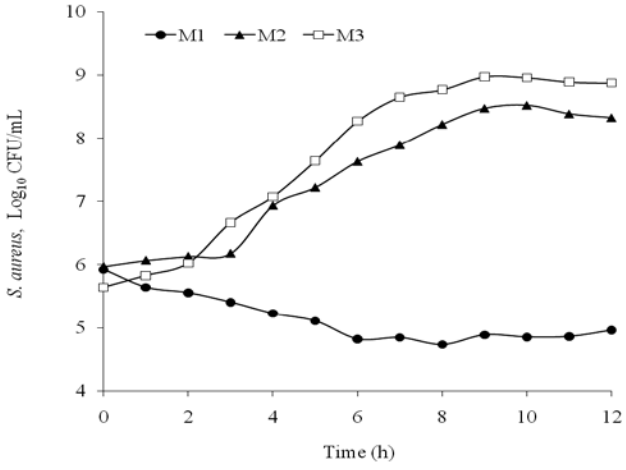

Figure 1 - Growth curves for *Staphylococcus aureus* after 12 hours of culture in coconut water (M1), coconut water enriched with 1% peptone (M2), and BHI (M3, positive control).

Gram-positive bacteria are nutritionally more demanding than Gram-negative bacteria as they need inorganic salts and organic and inorganic carbon for cell synthesis. Peptone is considered an excellent source of organic nitrogen for the synthesis of different groups of proteinogenic aminoacids, DNA, RNA and ATP (Tortora *et al*., 2000).

The Gram-positive species *S. aureus* was chosen for this study as it is considered the third most relevant cause of food-borne diseases worldwide (Tirado & Schmidt, 2001). The use of enriched coconut water as growth medium would reduce the cost of sanitary monitoring for *S. aureus* in food processing.

After 12 hours of culture, the average log values of the respective bacterial populations were 8.87533 (M3), 8.327000 (M2) and 4.959667 (M1) CFU/mL (Table I).

Table I - Average growth rates of *Staphylococcus aureus* (log CFU/mL) after 12 hours of culture in coconut water (M1), coconut water enriched with 1% peptone (M2), and BHI (M3, positive control).

Time (hour)	Log CFU/mL		
	M1	M2	M3
0	5.9253	5.9723	5.6407
1	5.6423	6.0677	5.8283
2	5.5550	6.1300	6.0237
3	5.4057	6.1790	6.6653
4	5.2250	6.9353	7.0737
5	5.1047	7.2200	7.6403
6	4.8183	7.6317	8.2710
7	4.8423	7.8960	8.6517
8	4.7310	8.2213	8.7693
9	4.8850	8.4760	8.9753
10	4.8497	8.5237	8.9600
11	4.8593	8.3873	8.8907
12	4.9597	8.3270	8.8753

Table II - Chemical composition of lyophilized coconut water (LCW) according to certificate issued by the Food Technology Institute (ITAL/CQA, 2010).

Carbohydrates (g)	**76.00**
Protein (g)	12.00
Moisture (g)	1.00
Total fat (g)	**4.00**
Saturated fat (g)	**2.46**
Monounsaturated fat (g)	
18:1 oleic acid (g)	0.12
Polyunsaturated fat (g)	0.47
Trans fat (g)	0.00
Cholesterol (mg)	0.00
Omega 3 (g)	0.02
Omega 6 (g)	0.02
Minerals - per 100g	
Sodium, Na (mg)	2240.00
Calcium, Ca (mg)	492.00
Iron, Fe (mg)	8.00
Phosphorous, P (mg)	430.00
Magnesium, Mg (mg)	510.00
Potassium, K (mg)	5170.00
Vitamins - per 100g	
Vitamin B1 (mg), thiamine	4.55
Vitamin B2 (mg), riboflavin	0.07
Vitamin B3 (mg), niacin (nicotinic acid and vitamin PP)	3.56
Vitamin B6 (mg), pyridoxine	0.13
Vitamin C (mg), ascorbic acid	16.51
Aminoacids - per 100g	
Aspartic acid (mg)	4.73
Glutamic acid (mg)	12.41

Table 3 - Comparison of average growth rates of *Staphylococus aureus* (log CFU/mL) after 12 hours of culture in coconut water (M1), coconut water enriched with 1% peptone (M2), and BHI (M3, positive control).

Growth medium	Average (Log CFU/mL)	Growth media compared	Difference (Log CFU/mL)
M3	8.875334	M3 vs. M2	0.548334
M2	8.327000	M2 vs. M1	3.367333
M1	4.959667	M3 vs. M1	3.915667

Lyophilized coconut water is translucid and almost colorless. The addition of peptone changes the appearance to turbid yellow, not unlike BHI (complex medium). Bacterial culture tends to darken growth media even further as carbohydrates participate in important chemical transformations (such as the Maillard reaction) and in caramelization of reducing and non-reducing sugars (Bobbio & Bobbio, 1995). Due to the negligible levels of nitrogen in natural coconut water, no darkening was observed in M1 throughout the experiment.

During lyophilization, the coconut water was at no time exposed to high temperatures, producing a final product with nutritional and sensory characteristics similar to coconut water *in natura*. The nutritional value, protein structures and vitamin contents (especially thermolabile vitamins) were preserved in the process.

Variance Analysis revealed significant differences between the three culture media (M1, M2 and M3) with regard to the growth rate of *S. aureus* (expressed in Log CFU/mL) after 12 hours of culture (p<0.0000603). The addition of nitrogen to the coconut water (M2) had a direct influence on the growth of *S. aureus*, yielding average growth rates similar to those of the positive control (M3). This finding differs from results published by Torres (2005) who found addictive-free coconut water diluted 50:50 with distilled water to be an adequate growth medium for *Escherichia coli* ATCC 25922.

S. aureus grew exponentially in both M2 and M3; however, the phase lasted 8 hours in the former (Figure 2a) and 6 hours in the latter (Figure 2b). Growth was negligible in M1 (Figure 2c). The lag phase tends to be longer when an inoculum from a rich and complex medium is seeded in a minimal defined medium, as the microorganism needs to synthesize a number of proteins which were readily available in the complex medium in order to generate the necessary cell components (Ingraham *et al*., 1997). The growth rate of *S. aureus* was 0.825 μ/h and the duplication time was 0.84 h (50.4 min) in M2. The corresponding figures for M3 were 1.260 μ/h and 0.55 h (33.0 min). The difference between M2 and M3 was not significant (p>0.855634) and may be attributed to the abundance, in the latter, of peptone, dextrose and calf brain, heart and muscle extracts.

In a study by Machoshvili & Penna (1991) for comparing alternative growth media for *S. aureus* (soy milk, alone or combined with caseine, cow milk, calcium carbonate, yeast, chocolate and sugar), the best adaptation to growth conditions and incubation was observed with plain soy milk at 33ºC (1.24 = μ/h; duplication time = 0.56 h).

CONCLUSION

Coconut water enriched with 1% peptone was shown to be a feasible alternative to commercially available growth media for *Staphylococcus aureus* culture.

REFERENCES

Agrianual. *Anuário da agricultura brasileira. Coco-da-baía*. FNP, Consultoria e Agroinformativos, p.286-292, 2006.

Aroucha, E.M. & Vianni, R. Água de coco por cromatografia liquida e pelo método titulométrico. *Revista Ceres*, v.49, n.283, p. 245-251, 2002.

Barbosa, H.R. & Torres, B.B. Nutrição, p. 90-101, in *Microbiologia básica*. Editora Atheneu, 1998.

Blume, H.; Vale- Filho, V.R.; Marques Jr, A.P. & Saturnino, H.M. Água de coco no cultivo de embriões bovinos. *Arq. Bras. Med. Vet. Zoot.*, v.50, n.4, p.395-399, 1998.

Bobbio, P.A & Bobbio, F.O. *Química do processamento de alimentos*. Editora Varela, 2ª edição, 151 p., São Paulo, 1995.

Costa, S.H.F.; Santos, R.R.; Rodrigues, A.P.R.; Celestino, J.J.H.; Matos, M.H.T.; Andrade, E.R.; Martins, F.S.; Dantas, J.K.; Ohashi, O.M. & Figueiredo, J.R. *In vitro* culture of ovine primordial follicles in media supplemented with coconut water. *Anim. Repr. Sci.*, v.3, n.4, p. 403-409, 2006.

Fagundes Neto, U.; Franco, L.; Tabacow, K.M.B.D. & Machado, N.L. Água de coco: variações de sua composição durante o processo de maturação. *Jornal de Pediatria,* v.65, n.1-2, p.17-21 1989.

FAO. Disponível em: <www.faostat.org.br>. Acesso em: 23 de Junho de 2006

Ingraham, J.L.; Maaloe, O. & Neidhardt, F.C. Microbial growth, p.136-157, *in* Perry, J.J. & Staley, J.T. (eds.), *Microbiology: dynamics and diversity*. Fort Sauders College Publishing, Fort Worth, 1997.

Machoshvili, I.A. & Penna, T.C.V. Growth rates of an enterotoxic strain of *Staphylococcus aureus* in soybean mil. *Rev. Farm. Bioq. Univ. S. Paulo*, v.27, n.1, p. 57-69, 1991.

Oliveira, R.V.; Nunes, J.F.; Salgueiro, C.C.M.; Cavalcante, J.M.M.; Moura, A.A. & Araújo, A.A. Avaliação morfológica de espermatozóides caprinos diluídos e congelados em meio à base de água de coco em pó (ACP-101) ou tris, corados por eosina-nigrosina e azul de bromofenol. *Ciên. Anim. Bras*., v.10, n.3, p. 862-869, 2009.

Pina, V.M.R.; Cavalcanti–Neto, C.C.; Holanda, G.M.L.; Freitas-Neto, L.M.; Santos-Junior, E.R.; Machado, P.P.; Paula-Lopes, F.F.; Lima, P.F. & Oliveira, M.A.L. Adição da água de coco (*Cocus nucifera*) ao meio de maturação de oócitos caninos. *Med. Veter*., v.1, n. 2, p. 42-49, 2007.

Smith, M. E & Bull, A.T. Studies of the utilization of coconut water waste for the production of the yeast *Saccharomyces fragilis*. *J. Appl. Bacteriol*., v.41, n.1, p.81-95, 1976.

Tirado, C. & Schimdt, K.; WHO surveillance programme for control of food-borne infections and intoxications: preliminary results and trends across greater Europe. *J. Infect*., v.43, n.1, p. 80-84, 2001.

Tortora, G.; Funke, B. & Case, C.L. *Microbiologia*. Artmed, 6ª edição, 827 p., Porto Alegre, 2000.

Torres, R.C.O. *Utilização de água de coco verde (*Cocos nucifera *L.) na composição de meios para cultura de Escherichia coli.* Tese de Doutorado em Ciência dos Alimentos, Universidade Federal de Santa Catarina, 89 p., Florianópolis, 2005.