Available online at www.sciencedirect.com
ScienceDirect
Bioresource Technology 98 (2007) 281–287
BIORESOURCE TECHNOLOGY

Coconut water as a medium additive for the production of docosahexaenoic acid (C22:6 n3) by *Schizochytrium mangrovei* Sk-02

Panida Unagul [a,b], Caetharin Assantachai [c], Saranya Phadungruengluij [c], Manop Suphantharika [c], Morakot Tanticharoen [a,b], Cornelis Verduyn [c,*]

[a] *BIOTEC, 113 Thailand Science Park, Phaholyothin Rd, Klong 1, Klong Luang, Pathum Thani 12120, Thailand*
[b] *School of Bioresources and Technology, King Mongkut's University of Technology Thonburi (Bangkhuntien), 83 Moo 8 Tientalay 25 Road, Thakham, Bangkhuntien, Bangkok 10150, Thailand*
[c] *Faculty of Science, Department of Biotechnology, Mahidol University, Rama 6 Rd, Bangkok 10400, Thailand*

Received 16 September 2005; received in revised form 10 January 2006; accepted 17 January 2006
Available online 23 March 2006

Abstract

The effect of coconut water (CW) on biomass and docosahexaenoic acid (DHA, C22:6 n3) formation by *Schizochytrium mangrovei* Sk-02 was studied in a yeast extract-diluted sea water medium. Optimal CW-level was ca. 33% (v/v), resulting in a biomass level of 28 g/l with a DHA-content of 20% (w/w) or 6 g DHA/l, almost 50% higher than in non-supplemented cultures at the same initial sugar level. Study on the growth-promoting effects of coconut water suggested that it could be (partially) mimicked by addition of trace elements; the fatty acids present in CW did not appear to be incorporated or effect fatty acid formation by the organism. CW-addition was also effective in media with other nitrogen sources such as casitone, peptone and tryptone. Its inclusion (at 50% v/v) increased biomass levels two-to-three-fold with concomitant increases in the DHA-level.

Keywords: Coconut water; Docosahexaenoic acid; Growth promoter; *Schizochytrium*; Yield

1. Introduction

Docosahexaenoic acid (DHA) is an essential polyunsaturated fatty acid (PUFA) and insufficient dietary intake has been implicated in a variety of human diseases (Horrocks and Yeo, 1999). The traditional source of DHA is fish oil but declining fish stocks, seasonal variability in oil composition, offensive odor and potential chemical contamination have stimulated research into alternative sources, in particular production by heterotrophic marine protists as *Crypthecondium cohnii* and thraustochytrids, including *Schizochytrium* and *Ulkenia* sp. (Ratledge, 2004; Sijtsma and de Swaaf, 2004; Ward and Singh, 2005). These organisms can be grown in complex media with carbohydrates as carbon and energy source. Yeast extract is a suitable nitrogen source for these organisms and has the benefit of significant vitamin levels. The aim of this study was to evaluate the effect of coconut water (CW) to improve DHA yields in a glucose–yeast extract-(diluted) sea water medium.

2. Methods

2.1. Microorganism and cultivation

Schizochytrium mangrovei sp. Sk-02 isolated in a mangrove forest was used in this study (Fan et al., 2001). Pure cultures were maintained on slants in solid GPY-medium containing 15 g/l each agar (Difco, USA) and artificial sea salts (ASS) (Sigma, USA) at 20 °C and sub-cultured every month. To prepare an inoculum, cells from a slant were streaked on a plate with the medium described above and

* Corresponding author. Tel.: +66 2 02201 5318; fax: +66 2 246 3026.
E-mail address: frcvd@mahidol.ac.th (C. Verduyn).

0960-8524/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2006.01.013

incubated at 25 °C for 24 h. Two circular wells (∅1 cm) were then aseptically cut in the agar with a large cork-borer and the plate was flushed with 10 ml of a 15 g/l solution of ASS. After two hours, zoospores were collected from the wells into a sterile tube, the OD_{660} was assayed and diluted to 7.0 by adding 15 g/l sterilized ASS. A 5% (v/v) inoculum was then transferred to a 250 ml Erlenmeyer containing 100 ml of standard medium containing (g/l) yeast extract (Difco, USA) 10, glucose 60 and ASS 15. Where indicated, the medium was dissolved in (diluted) mature coconut water (CW) obtained from a local manufacturer of canned coconut meat. In order to remove any larger particles, CW was first passed through a cheese cloth. CW contained monosaccharides (a total of 11 g/l fructose and glucose, see results), but practically it was difficult to adjust the total initial monosaccharides level to 60 g/l (as in the standard medium) for media with various levels of diluted CW, hence this was not pursued. Rather, controls were performed in which the glucose level in the standard medium was increased to 71 g/l by supplementation with the level of glucose and fructose found in undiluted CW. In addition, in an experiment where medium components were dissolved in undiluted CW, the amount of glucose added was reduced to 49 g/l to give an initial monosaccharides level of 60 g/l as in the standard medium. To obtain fatty acid-free coconut water, a freeze-dried aliquot (25 ml, yielding 1 g dry material) was extracted twice with 10 ml hexane. The hexane layers were combined and evaporated with a gentle stream of pure nitrogen gas at 40 °C. FAs were then assayed as described below. A non-extracted freeze-dried sample was used as a control and gave results identical to non-frozen coconut water. In order to identify possible growth-promoting factors in coconut water, the latter was replaced by addition of (combinations of) complete vitamin or trace element solutions (Verduyn et al., 1992), acetic acid or DL-malic acid, with the latter two at concentrations found in undiluted CW to standard medium. Concentrated vitamin solution contained (mg/l): biotin, 0.05; calcium panthotenate, 1; cyanocobalamin, 1; myo-inositol, 25; nicotinic acid, 1; para-aminobenzoic acid, 0.2; pyridoxine–HCl, 1; riboflavin 1, and thiamine–HCl, 1, whereas concentrated trace elements solution consisted of (mg/l): $CaCl_2 \cdot 2H_2O$, 4.5; $CoCl_2 \cdot 6H_2O$, 0.3; $CuSO_4 \cdot 5H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 3; H_3BO_4, 1; KI, 0.1; $MnCl_2 \cdot 4H_2O$, 1; $NaMoO_4 \cdot 2H_2O$, 0.4 and $ZnSO_4 \cdot 7H_2O$, 4.5. Where indicated, trace element and vitamin solutions were added to media at 1 ml/l.

Glucose was sterilized separately at 110 °C as a 50% solution in water or coconut water as required, whereas other medium components were sterilized together at 120 °C. Sterilization of media supplemented with CW did not lead to any precipitates or excessive browning. In an additional experiment, medium dissolved in pure CW was filter-sterilized (0.45 μm). This gave the same results as heat-sterilized medium. Flasks were shaken at 200 rpm and 25 °C for up to four days, Samples were taken regularly and centrifuged in 2 ml Eppendorf tubes (12,000 rpm for 10 min). The pellet was washed twice with distilled water and subsequently freeze-dried, whereas the supernatant was frozen at −20 °C for further analysis.

2.2. Fatty acid analysis

Freeze-dried biomass (ca. 10 and 20 mg dry weight) was weighed accurately and transferred to amber reaction vials. Two milliliter of 4% sulfuric acid in methanol was added, as well as C17:0 as an internal standard and butylated hydroxytoluene (BHT) as anti-oxidant at a final concentration of 0.1 g/l. After mixing on a vortex, the vials were incubated in a water bath at 90 °C for 1 h. After cooling, the mixture was extracted twice with a mixture of 1 ml of hexane and 1 ml of water. Further extractions did not increase recovery of fatty acid methyl esters (FAMEs). The extract was centrifuged (5 min at 3000*g*), and a small amount of solid anhydrous sodium sulfate was added to absorb water. The liquid phase was then injected on a Shimadzu GC-17A gas chromatograph equipped with a Omegawax 250™ (30 m × 0.25 mm) column (Supelco, USA), an auto-injector and flame ionization detector. Injector and detector temperatures were 250 and 260 °C, respectively, with helium as carrier gas at a linear velocity of 30 cm/s. Column temperature was held at 200 °C for 10 min, then increased to 230 at 10 °C/min and kept at this temperature for 14 min. The procedure was checked by methylation of pure palmitic and docosahexaenoic acid (Sigma, USA). Identity of FAMEs was initially confirmed with GC/MS; for routine analysis peak quantification was performed by comparison with four dilutions of a mixed PUFA-standard (no. 189-19) as well as pure DHA–FAME (both Sigma, USA).

2.3. Sugar and acid analysis

Free sugars were assayed by HPLC (Waters 2690 injector equipped with a model 410 differential refractometer) on a Sugar-Pak I (Waters, Milford, USA) column (300 × 6.5 mm) at 90 °C with a flow rate of 0.5 ml/min. The mobile phase consisted of double distilled water containing 50 mg/l EDTA. Before injection, samples were de-fatted by hexane extraction and subsequently filtered through a 0.45 μm filter. Injection volume was 50 μl. Organic acids were assayed by HPLC (Waters 290 injector) with a model 2996 photodiode array detector on an IC-Pak Ion-exclusion 50A column (150 × 7.8 mm) at 55 °C with a flow rate of 0.7 ml/min. The mobile phase consisted of 0.1% phosphoric acid (pH 2.0). Before injection (50 μl) samples were filtered through a 0.45 μm filter. Peaks that could be identified by this system were citric acid (retention time (RT) 4.42 ± 0.08 min), DL-malic acid (RT 5.18 ± 0.10), succinate (RT 6.02 ± 0.09), whereas fumaric acid and acetic acid overlapped (RT both 7.47 ± 0.10) and could not separated even when two columns were used in series. Hence acetic assayed was assayed by GC on a Shimadzu GC-17A instrument equipped with FID-detector and a 80/120 Carbopack B-DA/4% Carbowax 20M column (20 m × 0.2 mm). Column