Photosynthetic Bioconversion of Coenzyme Q_{10} Using Agrowaste Generated from Tobacco Biorefinery for Nonsmoking Applications: A Review

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ABSTRACT

An effective processing was established to recover protein without nicotine from tobacco for alternative applications as the foundation for tobacco biorefinery. Solanesol, an intermediate compound for the synthesis of coenzyme Q_{10} (CoQ_{10}) and vitamin K analogues, was recovered from tobacco biomass, an agrowaste generated after protein separation. CoQ_{10}, a potent antioxidative dietary supplement, was produced using photosynthetic *Rhodospirillum rubrum* ATCC 25852 by submerged fermentation supplemented with tobacco biomass hydrolysate. The stimulation effects on cell growth (shortened lag phase, accelerated exponential growth, and elevated final cell concentration) and CoQ_{10} production (enhanced specific CoQ_{10} content per unit cell weight) could be attributed to the solanesol in tobacco biomass. The significance of the present study is twofold. First, solanesol in the growth medium of *R. rubrum* provided the cells with the essential intermediate metabolites that could act like preassembled cassettes to be quickly configured into the finished product (CoQ_{10}). The ability of *R. rubrum* cells to accept these intermediate metabolites and bypass certain metabolic pathways makes *R. rubrum* an ideal candidate for CoQ_{10} bioproduction. Secondly, the fermentation process could be further optimized if the respective nutrient, dissolved oxygen, and light requirements for *R. rubrum* cell growth and CoQ_{10} production could be clearly elucidated.

Key words: CoQ_{10}, tobacco, biomass, photosynthesis, bioconversio

INTRODUCTION

Solanesol, an unsaturated polyisoprenoid alcohol containing all *trans* isoprene units[(1)], is known to possess anti-bacterial, anti-inflammation, and anti-ulcer activities[(2,3)]. Industrially, it is employed by the pharmaceutical industry as an intermediate compound for the synthesis (both chemically and biotechnologically) of metabolically active quinones such as coenzyme Q_{10} and vitamin K analogues[(4)]. The demand of solanesol continues to escalate since coenzyme Q_{10} entered the market as a dietary supplement largely due to its perceived benefits in providing relief for migraine headache sufferers[(5)], protecting people from Parkinson's disease and other neurodegenerative diseases[(6)], and improving blood pressure and long-term glycemic control for patients with type 2 diabetes[(7)].

Use of leaf hydrolysate in the growth media to stimulate microbial growth during fermentation is scarce but could be found in the literature. Yeh *et al*.[(8)] successfully demonstrated that substrates containing up to 20% (v/v) Chinese wolfberry hydrolysate notably shortened the lag phase with a significant increase in the final cell concentration of *Pediococcus acidilactici,* a probiotic strain widely used in the fermentation of dairy products, meats, vegetables, dough, fruit juices, and silage. Although no specific compounds responsible for such stimulating effects were identified in the aforementioned studies, a mild heat treatment to the leaves, similar to the tea-making process, appeared to be a mutual practice in attaining the growth stimulation effect[(8)].

Jiang *et al*.[(9)] reported that solanesol, an unsaturated polyisoprenoid alcohol employed by the pharmaceutical industry as an intermediate compound for the synthesis (both chemically and biotechnologically) of meta-

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bolically active quinones such as coenzyme Q_{10} and vitamin K analogues, could increase the yield of CoQ_{10} during fermentation. Our previous work[10] further identified that tobacco biomass acquired as a waste stream from the tobacco biorefinery process[11] contained approximately 0.1% solanesol on a dry weight basis. Growth medium supplemented with 20% (v/v) TBH was found favorable with regard to cell density and CoQ_{10} concentration. The stimulation effects on cell growth (shortened lag phase, accelerated exponential growth, and elevated final cell concentration) and CoQ_{10} production (enhanced specific CoQ_{10} content per unit cell weight) could be attributed to the presence of solanesol, the precursor of CoQ_{10}, in the tobacco biomass. The final yield of CoQ_{10} reached 20.16 mg/L in the fermentation medium supplemented with 20% TBH. It is the intent of this article to summarize the extensive efforts gone into bioconversion of CoQ_{10} using solanesol-containing tobacco biomass post protein recovery. Following the concept of biorefinery[10] (Figure 1), tobacco leaves could be transformed from an ill-imaged crop to a valuable natural resource for healthful ingredients without genetic engineering.

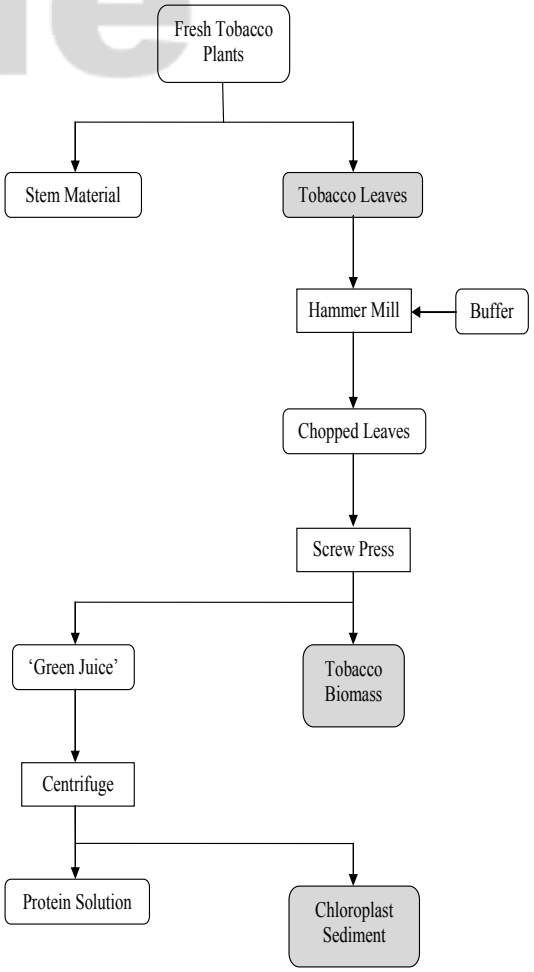

Figure 1. Schematic diagram of processes involved in tobacco biorefinery[10].

METHODOLOGIES AND APPROACHES

I. *Low Alkaloid Tobacco and Hydrolysate Samples*

Low alkaloid tobacco (*Nicotiana tabacum* cv. MD-609LA) containing an average nicotine level of 0.6 - 0.8 mg/g dry weight was grown on the University of Maryland Central Maryland Research and Education Center (Upper Marbolo, MD) during June - August of 2006 and 2007. Whole plants of tobacco were mechanistically harvested approximately 6 weeks after the transplant date, as indicated by the first appearance of a flower bud correspondent to tobacco plant physiological development that indicated the time for harvest. Each succeeding harvest after the first harvest occurred at ca. five week intervals following the first harvest. Freshly harvested plants were immediately misted with cool water, and transported within 3 h to the UM Food Bioprocess Engineering Laboratory (College Park, MD). The hydrolysates, namely tobacco biomass hydrolysate (TBH), alfalfa biomass hydrolysate (ABH), and spinach biomass hydrolysate (SBH), were prepared following the protocols specified by Yeh *et al.*[8]. Dried leaf samples (10.0 g) were placed in a 250 mL beaker containing 150 mL DI water and heated in a water bath at 75 - 80°C for 2 h. The hydrolysates were filtered through a Whatman No. 4 filter paper (Whatman Inc., Florham Park, NJ) and stored at -16°C before further uses.

II. *Extraction of Tobacco Protein*

Upon arrival, the leaves of MD-609LA (500 g) were separated from the stalks, rinsed clean with tap water, macerated, and homogenized in a Waring Blender for 3 min with the buffer solution containing known volumes of 5 mM Na_2EDTA (Fisher Scientific, Pittsburgh, PA) and 25 mM 2-mercaptoethanol (Sigma Aldrich, St. Louis, MO). HAc-NaAc, Na_2HPO_4-NaH_2PO_4, and Na_2HPO_4-KH_2PO_4 (respective effective buffering range at pH 3.6 - 5.8, 5.8 - 8.0, and 4.9 - 8.2) were investigated. The blended green tobacco slurry was stored at 4°C for 12 h before filtered through 4 layers of cheesecloth to separate the biomass from the juice, which was subjected to centrifugation at 30,000 ×*g* for 20 min at 4°C using a L7 ultracentrifuge (Beckman

Coulter, Palo Alto, CA). The green sludge containing chloroplast, starch, and other particulate materials was then cheesecloth-filtered from the soluble protein solution, i.e., the supernatant. The amber-colored supernatants were collected and kept at 4°C prior to further analysis. All extraction experiments were performed in triplicates.

III. *Solanesol Content Analysis*

The solanesol concentration in the samples was determined using a high performance liquid chromatography (HPLC) system (Shimadzu LC-2010A, Shimadzu Scientific, Columbia, MD) equipped with serial dual plunger pumps, a column oven, an autosampler, and an ultraviolet-visual (UV-VIS) detector at 215 nm. A Waters reversed-phase μBondapak C18 column (3.9 × 300 mm, 10 μm particle size) with a guard column (Waters, Milford, MA) was used. Three oven temperatures, namely 25, 35, and 45°C, were investigated. A solanesol standard obtained from Sigma-Aldrich (St. Louis, MO) was first analyzed at various concentrations (with and without the solvents employed) to establish a calibration curve between solanesol concentrations and the peak area in the chromatogram. The mixture of acetonitrile-isopropanol solution was used as the mobile phase, with the volumetric ratios (v/v) optimized to attain clear resolution of the chromatographic peaks. The resolution, defined as the peak separation divided by the mean peak width, was calculated using the Class VP 6.0 software supplied with the HPLC system. The analysis was carried out using injection volume of 10 μL with different flow rates (0.5, 0.6, and 0.8 mL/min).

IV. *Microorganism and Media*

Freeze-dried *R. rubrum* ATCC 25852 culture (ATCC, Manassas, VA) was hydrated with 9 mL sterilized water and inoculated into the ATCC medium, which contained (g/L): malic acid 2.5, yeast extract 1, $(NH_4)_2SO_4$ 1.25, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2 \cdot 2H_2O$ 0.07, K_2HPO_4 0.9, KH_2PO_4 0.6, ferric citrate 0.01, and EDTA 0.02. One milliliter trace element solution and 7.5 mL vitamin solution were also added to each liter of the ATCC medium. The trace element solution contained (g/L): ferric citrate 0.3, $MnSO_4 \cdot H_2O$ 0.002, H_3BO_3 0.001, $CuSO_4 \cdot 5H_2O$ 0.001, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.002, $ZnSO_4$ 0.001, EDTA 0.05, and $CaCl_2 \cdot 2H_2O$ 0.02. The composition of vitamin solution was (g/L): nicotinic acid 0.2, nicotinamide 0.2, thiamine•HCl 0.4, and biotin 0.008. After incubation under a tungsten lamp (100W, luminous flux = 1130 lumens) at 35°C for 48 h, the stock of *R. rubrum* was prepared by mixing the broth with sterilized glycerol (10% v/v) and stored at -70°C until used. The fermentation medium contained (g/L): malic acid 2.5, yeast extract 1.29, $(NH_4)_2SO_4$ 1.34, $MgSO_4 \cdot 7H_2O$ 0.2, K_2HPO_4 0.9, KH_2PO_4 0.6, ferric citrate 0.08, and EDTA 0.02. A 3-l stirred-tank bioreactor (Applikon, Schiedam, Netherlands) loaded with the fermentation medium specified above was sterilized at 121°C for 15 min. After cooling to scheduled temperature (35°C), the fermentation was initialized by injecting 5% (v/v) inoculum. The culture was incubated under a tungsten lamp at 35°C for 96 h. The pH was controlled at 6.9 ± 0.2 by gradual addition of 1 M HCl in response to pH variations. The agitation speed was 400 rpm.

V. *Analytical Methods*

The cell mass concentration was determined using a calibration curve correlating optical density at 620 nm and dry cell weight (DCW). The optical density at 620 nm was measured with a spectrophotometer (ThermoSpectronic, Rochester, NY). The DCW was determined after the culture broth was centrifuged at 10,000 rpm (9159.4 ×*g*) for 30 min under 4°C using a Beckman Coulter L7 Ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) equipped with a Type 70.1 Ti rotor to precipitate the suspended cells. For assaying the CoQ_{10} content, the celLytic B (Sigma-Aldrich Co., St. Louis, MO) solution of 0.5 mL was added to the cell pellet acquired by the centrifugation procedures described above. After 30 min incubation, a solvent mixture of propanol and hexane (1 : 2 v/v) was added to the solution for cell lysis under vigorous mixing. The solvent phase, as well as that obtained by second extraction from the aqueous phase, were combined and evaporated to dry using a speed vacuum concentrator Rota-vapor (Heidolph, Heizbad, Laborota 4001, Germany). The dry residue was dissolved in ethanol and applied to a high-performance liquid chromatography (HPLC) system (LC-2010A, Shimadzu, Tokyo, Japan) with a μBondapak C18 (10 μm, 3.9 × 300 mm, Waters, Milford, MA) coupled to a UV detector (Waters 486). The column was eluted with ethanol and methanol (9 : 1, v/v) at a flow rate of 0.6 mL/min and a chromatogram was obtained by monitoring the absorbance at 275 nm. The CoQ_{10} content was identified and quantified by comparing the peak areas in the chromatogram with a calibration curve prepared by known concentrations of authentic CoQ_{10} standard (Sigma-Aldrich, St. Louis, MO).

FERMENTATION OPTIMIZATION

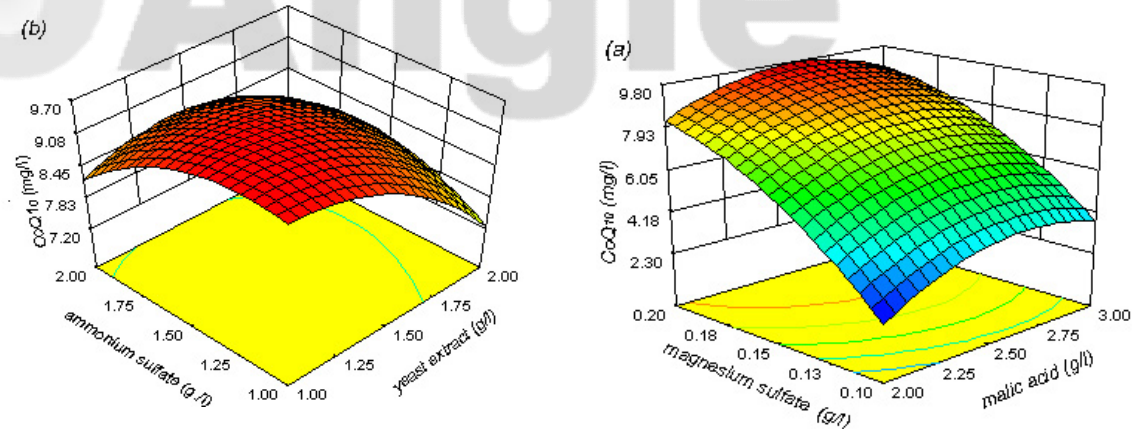

Figure 2. 3-D response surface contour plots showing the experimental factors and their mutual interactions on CoQ10 extraction: (a) Y = f (X1, 1.29, 1.34, X4, 0.08); (b) Y = f (2.50, X2, X3, 0.20, 0.08). Y, content of CoQ10 (mg/L); X1, Malic acid (g/L); X2, Yeast extract (g/L); X3, Ammonium sulfate (g/L); X4, Magnesium sulfate (g/L); X5, Ferric citrate (g/L).

Five variables X_1 (malic acid), X_2 (yeast extract), X_3 (ammonium sulfate), X_4 ($MgSO_4 \cdot 7H_2O$), and X_5 (ferric citrate) were selected and further optimized using Box-Behnken design in RSM to determine the optimal values for each of the factors[12]. Based on these observations, three-dimensional surface plots were constructed to determine the levels of the processing variables that contribute to reaching the optimal yield of CoQ_{10} (Figure 2). The interactions between two variables were depicted by keeping the other variables at their optimal levels for CoQ_{10} production. It could be clearly seen that, when yeast extract, ammonium sulfate, and ferric citrate were respectively set to 1.29, 1.34, and 0.08 g/L, the highestCoQ_{10} production should locate at the malic acid and magnesium sulfate ranging 2.05 - 3.00 g/L and 0.18 - 0.20 g/L, respectively (Figure 2a). By setting malic acid, magnesium sulfate, and ferric citrate to 2.50, 0.20, and 0.08 g/L, the optimal CoQ_{10} yield should be achieved with yeast extract in the range of 1.00 - 1.36 g/L and ammonium sulfate 1.00 - 1.62 g/L (Figure 2b).

SUPPLEMENTATION WITH TOBACCO BIOMASS HYDROLYSATE

Since production of CoQ_{10} by *R. rubrum* is strictly growth-associated, which means the cells must be lysed in order to release CoQ_{10} for further separation and purification. It would be economically informative if the accumulation of CoQ_{10} in the fermentation broth could be profiled. At 36 h into the fermentation, the amount of CoQ_{10} produced in the medium supplemented with 20% TBH reached 12 mg/L, already exceeding the final CoQ_{10} concentration reached in the fermentation medium (less than 10 mg/L) or those supplemented with ABH or SBH[13]. The accumulation of CoQ_{10} in TBH-supplemented medium reached more than 20 mg/L after 96 h of fermentation, more than twice those reached in the fermentation medium. The specific CoQ_{10} content ached in fermentation supplemented with 20% TBH was found the highest and significantly higher than the other conditions investigated (Figure 3), indicating that *R. rubrum* cells not only grew faster and reached higher cell density but also produced more CoQ_{10} per cell when TBH was present in the growth medium than without.

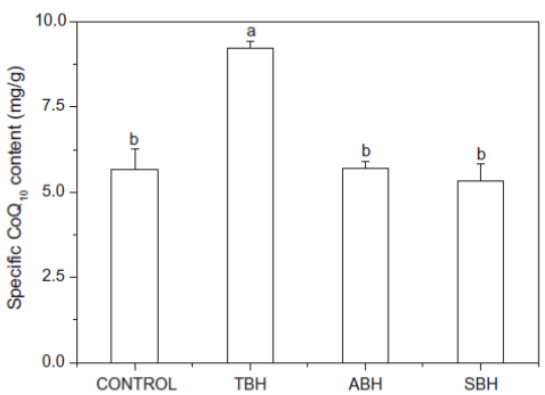

Figure 3. Effect of tobacco biomass hydrolysate (TBH), alfalfa biomass hydrolysate (ABH), and spinach biomass hydrolysate (SBH) on the specific CoQ_{10} content. Data are shown as mean ± SD (n = 3). Means with the same letter are not significantly different ($p < 0.05$)[13].

ENHANCED YIELD BY IMPROVING CELL LYSIS

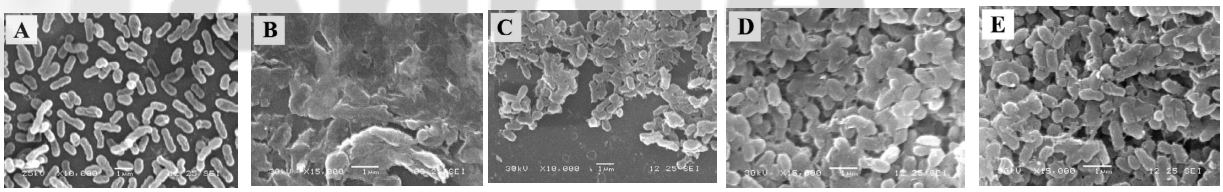

Figure 4 . Scanning electron micrographs of microbial cells (a) without any treatment; (b) after ultrasound treatment; (c) after repeated freezing and thawing; (d) after grinding; and (e) after acid-heat treatment[14].

Lysis of microbial cells was achieved by treating the cells with sonication, freezing and thawing, grinding and acid-heat methods. It is evident that cell lysis by acid-heat treatment proved to be superior to other methods in terms of CoQ_{10} yield, which was significantly higher than that achieved by the other methods. While sonication is commonly employed in research laboratories for cell disintegration, it is also known to generate microscopic bubbles. These transient cavities are thought to create high-shear gradients by microstreaming, with most cavitational effects only observed close to the vibrating surface. Therefore, disintegration of cells might be incomplete, as evidenced by SEM observations[14] (Figure 4b).

Moreover, the significance of the findings from the present study is twofold. First, the presence of solanesol, which is a precursor for CoQ_{10}, in the growth medium of *R. rubrum* provided the cells with the essential intermediate metabolites that could act like preassembled cassettes (or cartridges, modules etc.) to be quickly configured into the finished product (CoQ_{10}). The ability of *R. rubrum* cells to accept these intermediate metabolites and bypass certain metabolic pathways makes *R. rubrum* an ideal candidate for bioproduction of CoQ_{10}. Secondly, unlike other fermentation metabolites such as ethanol or acid that inhibit cell growth, solanesol instead stimulated (or at least did not inhibit) the growth of *R. rubrum* cells, suggesting that the fermentation process could be further optimized if the respective nutrient, dissolved oxygen, and light requirement for *R. rubrum* cell growth and CoQ_{10} production could be clearly elucidated.

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