Optimized Submerged Batch Fermentation for Co-enzyme Q Production by *Candida glabrata* Using Renewable Substrates

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ABSTRACT
CoQ_{10} is an endogenous antioxidant that is present in all tissues, varying in quantity and function, and is used to reduce oxidative stress, marketed as a dietary supplement and used in cosmetic formulations. This study aimed to investigate the production of CoQ_{10} by *Candida glabrata* using an optimized medium, compound of agro-industrial wastes (whey and corn steep liquor), evaluated the production, its cytotoxic effect and antioxidant potential. The production of CoQ_{10} was initially performed in synthetic medium and production medium optimized, with replacement of glucose by whey and peptone by corn steep liquor as substrates. Concentrations were established by experimental design (CCD) 2^2. The optimized medium in the condition 4 (40% whey and 20% corn steep liquor) presented the higher biomass yield of $9.1g/L^{-1}$ and the CoQ_{10} production was confirmed after analysis comparative of the retention time of the standard with the sample, in terms of quantification CoQ_{10} obtained a value yield of 150µg/g. Determination of cytotoxic activity was performed by the method of cell viability *in vitro* using the dye MTT, in the concentration of CoQ_{10} 25µg/mL, showed no cytotoxic activity on murine macrophages cells. The antioxidant potential was determined by the DPPH method, results of antioxidant activity showed a high antioxidant power of 73%. *C. glabrata* demonstrated to be an excellent producer of CoQ_{10} by biotechnological process with prospects for their use in industrial processes in the area of cosmetics.

Keywords
Candida glabrata, agro-industrial wastes, CoQ_{10}, potential antioxidant, cytotoxicity.

1. INTRODUCTION
The growth of the industrial sector has brought many benefits to the population, providing considerable improvement in the quality of life in the world. However high productivity it led to the rapid decline of natural resources and at the same time, the generation of large amounts of industrial wastes. The reuse of agro-industrial wastes, after the detection of their potential, are currently being considered an alternative that can contribute to the diversification of products, reduction of biomolecules production costs for providing alternative raw materials in fermentation processes with different types of microorganisms in biotechnological processes (Accorsini, 2012; Ortiz et al., 2015).

The production of secondary metabolites by biotechnological processes applied to the cosmetics industry, combined with the average increase of life of the population creates prospects for the world market. As a result of that hope, there is now a great demand for products for rejuvenation. This search led to the emergence of new products applied in the field of cosmetics, where substances that produce a biological effect of cell renewal in the tissue presenting benefits and therefore satisfies the high demands of society (Silva, Ferrari, 2011).

However, one of the main reasons cited by researchers as responsible for the aging process is the imbalance of antioxidant defense mechanism of our body caused by oxidative stress (Novaes et al., 2014).

The oxidative stress can be caused by different factors extrinsic as UV rays or intrinsic as metabolites generated in cell metabolism. Therefore, the cosmetics have reducing properties of skin aging signs. CoQ_{10} is an endogenous antioxidant which is present in all tissues, varying the amount and function, and is used to combat oxidative stress. The levels of this coenzyme are reduced with age and in patients with chronic diseases (heart, muscular dystrophy, Parkinson's, cancer, diabetes and HIV-AIDS) (Bentingr et al., 2007; Marsical et al., 2014).

The literature deals only with the production of CoQ_{10} by biotechnological processes. However, the application process is restricted and few information can be found about CoQ_{10} in use in cosmetic (Yen et al., 2009; Ha et al., 2009).

The optimization through experimental design is a common practice in biotechnology to select the best compounds of medium and the culture conditions, considering that the medium are responsible for about 30% of the total production value (Makkar, Cameotra, 2002; França et al., 2015).

This study aimed to investigate the potential biotechnological production of Coenzyme Q_{10} by *Candida glabrata* (1556), using a medium optimized economically viable the basis of agro-industrial wastes (whey and corn steep liquor), as nutritional sources (carbon and nitrogen) and evaluate its toxicity and its antioxidant potential for applications in the cosmetics industry.

2. DATA AND METHODOLOGY

2.1 Microorganism

Microorganism: *Candida glabrata* (UCP 1556) isolated from the semi-arid land (city Serra Talhada, Pernambuco, Brazil), was kindly provided by Collection Research Center of cultures in Environmental Sciences and Biotechnology (NPCIAMB) of the Catholic University of Pernambuco (UNICAP), registered in the World Federation Culture Collection - WFCC.

2.2 Agro-industrial Substrates

The production medium was composed by agro-industrial wastes: corn steep liquor (CSL), a byproduct of corn processing industry and whey (W) from the dairy industry of São Bento do Una, Pernambuco, Brazil.

2.3 Maintenance medium

The medium for maintaining the yeast was Yeast Mould Agar (YMA), with the following composition: yeast extract 3g; peptone 5g; malt extract 3g; glucose 10g; agar 15g; Distilled water 1000 ml, pH 5.8 and incubated for 48h at 28°C.

2.4 Optimization of biomass production by *C. glabrata* (UCP 1556)

The control medium used was that described by Natori et al., (1978), composed by: Yeast extract (1g/ml), peptone (1 g/ml), glucose (2 g/ml), $CaCO_3$ (0.12 g/ml) and $MgSO_4$ (0.03 g/ml). The research strategy chosen for optimization was from the formulation of an experimental design of type Central Composite Design (CCD) 2^2, formulated with different concentrations of agro-industrial wastes, (W) replacing glucose as carbon source and (CSL) replacing peptone as a nitrogen source. The levels of the experimental design are shown in Table 1. The yeast extract and mineral concentrations in the medium were constant in all conditions of the experimental design.

Table 1 - Levels of experimental design (CCD) 2^2 for optimized production of biomass by *C. glabrata* (1556)

Factor	Levels				
	-1.41	**-1**	**0**	**1**	**1.41**
Whey$_{(\% v/v)}$	15.9	20	30	40	44.1
Corn steep liquor $_{(\%v/v)}$	7.95	10.0	15	20	22.05

Statistical analysis was demonstrated in standardized Pareto diagram for concentrations used of CSL and W, using as response variable the biomass production. Estimates of the effects that were statistically significant are indicated by dashed line. The magnitude of each and the efficiency is represented by column and transverse line corresponding the value p = 0.5, indicating statistical significance and efficiency. The levels of the experimental design (CCD) 2^2 were encoded as: -1, +1 and 0 (zero is regarded as the center point) and the axial points (+1.41 and -1.41) were calculated using the following formula:

$$X_i = \frac{x_i - x_0}{\Delta x_i}$$

Where Xi is the encoded value of the independent variable, x_i is the real value of the independent variable, x_0 is the real value of the independent variable at the center point and Δx_i is the variable value. The significance of the effect was tested by analysis of variance (ANOVA), and statistically analyzed from the data of the experimental design, including Pareto diagram, the Statistic Statsoft (US) version 7.0 software.

2.5 Preparation of the inoculum

The inoculum was standardized transferring cells of *C. glabrata* (UCP 1556) to the Cald Yeast Mold (CYM), maintained at temperature of 28°C and 150rpm for 24 hours to obtain 10^7 cells\ml.

2.6 Biomass production by *C. glabrata* (UCP 1556)

The cultures for production of biomass were performed in Erlenmeyer flasks with 1L capacity, containing 500mL of the medium control and other 12 flasks with 500mL of medium of production (whey and corn steep liquor) of according concentration to the experimental design, inoculated suspension of 10^7 cells/ml. The bottles were kept under orbital agitation 150rpm, incubated for 72h at 28ºC. After this period samples were centrifuged at 4.000g, followed by filtration (120F silkscreen nylon membrane) to separate the cells of metabolic liquid. The biomass obtained was washed with deionized water, frozen and lyophilized kept in a desiccator until constant weight for quantification of dry biomass, estimated by gravimetrically. In optimized condition were collected aliquots at 4h, 8h, 12h and then every 24hours totaling 72 hours.

2.7 Determination of the glucose consumption and pH

The consumption of glucose was measured by enzymatic colorimetric method (Labtest) using D-glucose as standard and absorbance at 500nm in a spectrophotometer. To determine the pH was used potentiometer Orion (Model 310).

2.8 Extraction of CoQ_{10}

The biomass with 40g weight was kept under reflux at 90^0C for 20 minutes, treated with 25mL of distilled water, 75mL methanol, 2.5g pirogaloll and 10g of sodium hydroxide, to saponification. The saponified biomass, after cooling with ice, was transferred to separator funnel (500mL), submitted to extraction with 100mL of hexane and shaking manually for 20 minutes, leaving to stand until separation of the two phases. The upper phase was poured into Erlenmeyer flasks of 250 mL capacity and this process was repeated two more times. The upper phases were washed three times with distilled water and evaporated to dryness (rotaevaporator) under 40^0C temperature. Then were suspended in 2ml of acetone, concentrated to 25% volume and subjected to thin layer chromatography for pre-purification (Okada et al., 1995).

2.9 Identification of CoQ_{10} - Thin layer chromatography (TLC)

In this analysis we used a 20x20cm plate of silica gel and hexane as solvent system to separate CoQ_{10}. The pattern of CoQ_{10} and the sample were applied to the plate with the aid of a capillary. The identification of CoQ_{10} was evidenced for comparison of the retention time (R_T) of the standard with the sample. This region was removed with a spatula and placed in a Becker of 100 mL capacity, added 20 mL of acetone and placed under stirring for 30 minutes at the end of that period was centrifuged at 3.000g for 5 minutes. The supernatant was collected and evaporated at 40^0C temperature. The extracts containing the CoQ_{10} was suspended in 0.5 mL of acetone and submitted for identification by HPLC (Okada et al., 1995).

2.10 High performance liquid chromatography (HPLC)

The purified ubiquinone CoQ_{10} was analyzed by high performance liquid chromatography (HPLC) Alliance model 2695 and identified by comparison of retention times of the sample with the standard of ubiquinone CoQ_{10} and quantification performed after construction of the calibration curve with six different concentrations (0.05 to 0.5mg/ml) using the peak area to calculate the concentration (Rodrigues-Amaya, 1999). Analyzes were performed using a SUNFIRE C18 column (4.6x250mm) under the following conditions: acetonitrile: isopropanol (55%: 45%), flow: 0.6ml/min, UV detection: 270nm and temperature of 30^0C oven.

2.11 Determining the cytotoxic activity of CoQ_{10}

The cytotoxic activity was performed using the method MTT 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (Alley et al., 1988; Mosmann, 1983).

Human tumor cell strains were used NCI-H292 (human pulmonary mucoepidermoid carcinoma), HEp-2 (human laryngeal carcinoma) and J744.A1 (murine macrophage) maintained in DMEM culture. The medium were supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin and streptomycin). The cells were maintained in an incubator at 37°C in a humid atmosphere enriched with 5% CO_2.

The NCI-H292 cells, HEp-2 cells (10^5 cells/mL) and J744.A1 ($17{,}5x10^4$ cells/mL) were plated in 96 well plates and incubated for 24h. Then the samples dissolved in DMSO (1%) were added to the wells at final concentration of 25µg/mL. The drug doxorubicin (5µg/mL) was used as standard. After 72h of reincubation, was added 25µL of MTT (5mg/mL) and after 3h incubation, the culture medium with MTT was aspirated and 100µL of DMSO was added to each well. The absorbance was measured in a microplate reader at a wavelength of 560nm. The experiments were analyzed in GraphPad Prism5.0 program. Each sample was tested in triplicate.

2.12 Determination of antioxidant activity "Scavenger" of DPPH radical

The radical DPPH (1,1-diphenyl-2-picryl hidrazila) is considered a stable radical and has a maximum absorbance at 517nm. When this compound receives electrons hydrogen or a radical of an oxidizing agent or of a free radical to become a stable compound, results in loss of absorbance, from violet to yellow. To determine the "scavenger" of DPPH method as proposed by Brande-Williams et al., (2005). The flavonoid quercetin was used as a positive control for the experiment. Added to 1 mL of DPPH 0.3mM reagent only the samples. To the control was prepared a solution containing ethanol (2.5mL) and 3mM DPPH (1mL) and as white control 3.5mL of ethanol. Last 30 minutes, the absorbance was read at 517nm and calculated as a percentage of antioxidant activity using the equation:

AA% = 100 - (Abs Sample – Abs White x 100) / (Abs Control - Abs White)

The assay was performed in triplicate and the concentration capable of inhibiting 50% of DPPH radical (IC^{50}) was calculated by linear regression, and expressed as media ± standard deviation (SD) where the X axis represents concentration and the Y axis the percentage of activity antioxidant (AA%). Statistical analyzes were performed using GranphPad Prisma program.

3. RESULTS AND DISCUSSION

3.1 Optimization of biomass production by *C. glabrata* (UCP 1556)

The control medium proposed by Natori et al., (1978) showed a production $5.1g/L^{-1}$ of biomass. The strategy chosen for optimization studies was to formulate an experimental design of the type CCD 2^2 (Table 1). The result of this experimental design shows that the condition 4, with optimized medium, W (40%) and CSL (20%) showed the best biomass production $9.1g/L^{-1}$ (Table 2).

The effects of whey and corn steep liquor for the production of biomass and the interaction between them were analyzed and are shown on the Pareto diagram (Figure 1). All variables were significant from a statistical point of view. The independent variables, corn steep liquor and whey in quadratic function and the interactions between them showed a statistically significant positive effect of favoring the production of biomass in the culture (Figure 1). Studies to evaluate the growth conditions, fermentation and medium optimization of components for lactic acid production by *Lactobacillus coryniformis* were performed and obtained in the best condition a biomass production $5.1g/L^{-1}$ (Jamarillo et al., 2015).

Table 2 - The planning results (CCD) 2^2 for biomass production by *C. glabrata* (UCP 1556)

Conditions	Whey (%)	Corn steep liquor (%)	Biomass ($g.L^{-1}$)
1	20	10	3.30
2	20	20	3.70
3	40	10	6.9
4	40	20	9.10
5	15.90	15	3.20
6	44.1	15	7.50
7	30	7.95	3.61
8	30	22.05	3.98
9	30	15	5.30
10	30	15	5.87
11	30	15	5.61
12	30	15	5.69

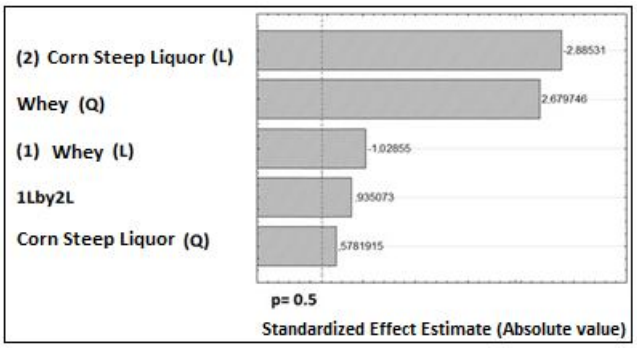

Figure 1 - Pareto diagram of standardized effects experimental design (CCD) 2^2, having as factors the W and CSL and as biomass production with response variable.

Athiyaman and Sankaranarayanan (2014) conducted a study biotechnological to production of CoQ_{10} by *R. glutinis* in optimized medium the basis hydroxy-benzoic acid (PHB) and soybean oil (7.78%) using response surface methodology, the culture conditions promoted an increased significative for production of CoQ_{10}. Production increased considerably from $10mg/L^{-1}$ (control medium) to $39.2mg/L^{-1}$ (optimized medium).

3.2 Kinetics of growth of optimized medium by *C. glabrata* (UCP 1556)

C. glabrata showed a maximum production of cell biomass in the control medium $5.1g/L^{-1}$ and the production medium optimized $9.1g/L^{-1}$ after 72 hours of cultivation, it became evident that the production medium the basis of agro-industrial wastes significantly increased cell biomass production compared to the control medium, however the two assays showed similar values throughout the culture, in the first 4 hours a pH around 4.0 and at the end of 72 hours a slightly alkaline pH around 6.0 (Figure 2A). *C. glabrata* in the control medium obtained a glucose consumption of 63% and in the production medium optimized (condition 4) 80% after 72 hours of culture (Figure 2B).

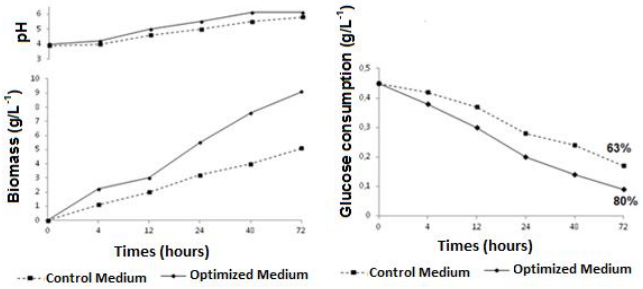

Figure 2 - (A) Determination of glucose consumption; (B) pH and biomass production by *C. glabrata* (UCP 1556) in the control medium and in the production medium optimized.

Cazetta and Celligoi (2006), using a biotechnological process for the production of cell biomass in strains *C. lipolytica*, *R. mucilaginosa* and *S. cerevisiae* in production medium containing, molasses 50% and vinasse 50%, obtained a glucose consumption above 50%, and biomass values of 6.9; 2.63 and $7.49g/L^{-1}$, respectively.

3.3 Identification and quantification of CoQ_{10}

CoQ_{10} is an important molecule industrially by presenting applications nutraceuticals and cosmeceuticals. CoQ_{10} is produced by microbial fermentation and the process requires the use of strains with high productivity and yield of CoQ_{10} (Ranadive et al., 2014). Analyzing the chromatograms it was observed identification of CoQ_{10} comparing the retention times (R_T) of standard with the sample containing extracts of CoQ_{10} produced by *C. glabrata*, showing values similar between the R_T of standard 5.371 and R_T sample 5.372. In terms of quantification CoQ_{10} presented yield of 150µg/g (Figure 3).

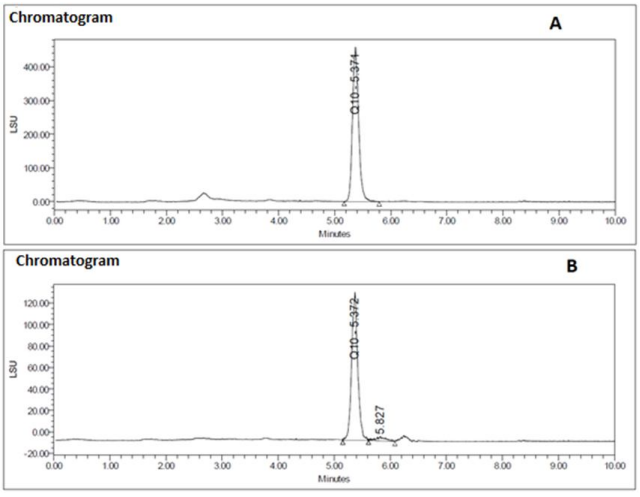

Figure 3 - Chromatograms. (A) Standard solution of CoQ_{10} at a concentration of 1mg/ml; (B) sample containing purified extract of CoQ_{10} produced by the *C. glabrata* at a concentration of 1mg/ml.

3.4 Analysis of the antioxidant activity of CoQ_{10}

The results of the assay for the determination of DPPH scavenging ability are expressed in IC^{50} - µg/mL, indicating the extract concentration required to reduce by 50% the amount of free radicals. The scavenging capacity of free radical DPPH of CoQ_{10} produced by *C. glabrata* in optimized medium (condition 4) was 6.90µg/mL. According Nguma (2010), the lower the concentration, greater the antioxidant capacity of the sample. Nguma (2010), held a study evaluating the antioxidant capacity of BHT (Butyl hydroxy toluene), ascorbic acid and gallic acid, the assay values were 17.2; 9.2 and 2.4µg/mL, respectively. We can say that the sample of CoQ_{10} produced by *C. glabrata* has greater antioxidant potential in relation to the BHT and Ac. ascorbic, which is a very promising result to the industry.

3.5 Cytotoxic potential of CoQ_{10}

The CoQ_{10} extract produced by *C. glabrata* at condition 4 with optimized medium (40% W and 20% CSL) was tested for cytotoxic activity on human tumor cells (NCI-H292 and Hep-2) and murine macrophage cells (J774.1). An intensity scale was used to assess the cytotoxic potential of the samples tested. Samples with no activity (1 to 20% inhibition), with little activity (cell growth inhibition ranging from 20 to 50%), with moderate activity (cell growth inhibition ranging from 50 to 70%) and much activity (inhibition growth varying from 70 to 100%) (Fouche et al., 2008). The results showed that at a concentration of 25µg/mL CoQ_{10} produced by *C. glabrata* (UCP 1556) showed no inhibition activity on cells of murine macrophages (J774.1) with 0%. However in human tumor cells (NCI-H292 and Hep-2) it was observed little activity inhibition with values of 20.98 and 13.39%, respectively.

4. CONCLUSION

This study produced by biotechnological process CoQ_{10} by *Candida glabrata (*UCP 1556) in optimized medium compound by agro-industrial wastes. The interactions between the substrates (whey and corn steep liquor) emerge as a viable alternative for the production and reducing costs in the production of CoQ_{10}. *C. glabrata* demonstrated to be an excellent producer of CoQ_{10} with high antioxidant power and on murine macrophage cells showed no activity cytotoxic. CoQ_{10} produced in this work demonstrates prospects for their use in industrial processes in the area of cosmetics.

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