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# *Xanthophyllomyces dendrorhous* physiological stages determination using combined measurements from dielectric and Raman spectroscopies, a cell counter system and fluorescence flow cytometry

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ABSTRACT

Dielectric spectroscopy, Raman spectroscopy, a cell counter system and fluorescence flow cytometry were used to monitor the yeast *Xanthophyllomyces dendrorhous* in bioreactor batch cultures. From the dielectric spectroscopy signal, it was possible to determine the maximum biomass growth, the viability of the cell and the time when the substrate was consumed. Raman spectroscopy allowed to measure intracellular carotenoids production. Cell vacuole metabolic activity was monitored from fluorescence flow cytometry and the cell counter allowed detect cell volume variability along the fermentation. The information obtained from the sensors measurements allowed us to suggest and characterize different physiological states of the yeast *X. dendrorhous*.



## 1. Introduction

In a bioprocess, nutritional and operational conditions are manipulated in the bioreactor to guarantee high metabolite concentrations produced by microorganisms. Metabolite production in a bioreactor is the result of monitoring and controlling conventional variables such as: temperature, pH, and dissolved oxygen. These variables provide important information about physicochemical conditions surrounding the microorganism. However, promoting the production of a specific sub-product requires going beyond the use of conventional sensors, and demands to be aware of the physiological state of microorganisms in the culture. The lack of cellular viability influences the rate, the yield and the quality of the produced metabolite [1]. The cellular components having effect on the fermentation activity and the characteristics of a cell population reflecting changes in the culture conditions are defined as the physiological state of a microorganism [2]. To detect this kind of changes in a bioprocess it is required to monitor multiple variables such as size, morphology and viability of the cells using advanced non-conventional instrumentation.

Technology advances has allowed the design of new sensors to extract important characteristics of the cells. For instance, spectroscopic techniques have been used to monitor in-situ biotechnological processes [3]. Dielectric spectroscopy measures the cell membrane permittivity when it is polarized by an electrical field [4] providing information about cell viability, changes in cell volume, cell shape, and cell concentration [5,6]. Multi-wavelength fluorescence spectroscopy has been applied to monitor *E. coli* biomass concentrations and glucose depletion in a fed-batch culture [7]. Lipstatin productivity was enhanced by monitoring with Near-infrared spectroscopy the critical nutrient ratio of linoleic acid to ammonia in a *Streptomyces toxitricini* fermentation [8], it has also been used to monitor bioethanol production [9]. Multiple-frequency impedance measurements were applied to monitor yeast cell lysis [10]. A multi-sensor system was designed

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to determine in-situ nutrients, metabolites, cell density and pH of *Saccharomyces cerevisiae* or *Lactobacillus acidophilus* cultures [11]. Cell size and morphology are important variables that can be associated to the cell physiological state. The physiological response of a yeast population of *Saccharomyces cerevisiae* was determined from cell size distribution analysis using a cell counter [12]. Fluorescence microscopy and fluorochromes have been applied to stain cells and detect mitochondrial and vacuolar activity in yeast [13]. Flow cytometry has been used to analyze metabolites, cell viability and the physiological state of microorganisms [14–17]. Raman spectroscopy was applied to simultaneous measure nutrients, metabolites or subproducts and cell density in pharmaceutical processes [18], for instance the pigment astaxanthin [19].

The yeast *Xanthophyllomyces dendrorhous* formerly (*Phaffia rhodozyma*) synthesizes astaxanthin as its main carotenoid [20]. The astaxanthin is the main pigment in crustaceans, salmonids and flamingoes, among other organisms [21]. It has been used in aquaculture and poultry industries, because of these animals cannot synthesize this carotenoid and it must be included into their feed, not only provides an appealing color to the consumer, but also it operates as a nutritional component for growth and reproduction [22]. The astaxanthin has antioxidant activity, which enhances immune response in humans [23] and it can prevent some kinds of cancer [24]. This carotenoid can be produced by synthetic chemical technology; however, the subproducts generated may have side effects for the consumer; therefore, astaxanthin obtained from microbiological sources can be considered as a potential alternative. Worldwide, the astaxanthin is the third most important carotenoid, right after β-carotene and lutein, and it is estimated that in 2018 its market value will exceed the $250M USD [25], therefore, strategies to increase astaxanthin synthesis and extraction out of the cell have been considered [26–28].

It is expected to generate important information about the physiological state of microorganisms from monitoring variables such as viability, morphology, cell volume size and metabolite production using Raman and dielectric spectroscopy, a cell counter and fluorescence flow cytometry techniques. However, in most cases these non-conventional sensors have been used separately reflecting only specific characteristics of the cell. The greatest potential of advance instrumentation will be obtained from analyzing as a whole the information acquired from diverse sensors and correlate them with the physiological states of the cell. Therefore, in this contribution we analyze as a whole, data obtained from diverse advanced sensors and demonstrate that it can be associated with the physiological state of the yeast *X. dendrorhous*.

## 2. Materials and methods

### 2.1. Microorganism and culture conditions

#### 2.1.1. Microorganism

The astaxanthin-overproducing mutant strain 25-2 was obtained from the wild type *X. dendrorhous* ATCC 24202 [29]. The strain was conserved in 2 mL cryogenic vials Corning® containing sterile glycerol Sigma-Aldrich® and culture media with cells in a ratio of 1:1 and stored within an ultra-freezer Thermo Scientific® 8000 at a temperature of −80 °C.

#### 2.1.2. Culture media

The culture media contained (g/L): 20.0 glucose, 3.0 $KH_2PO_4$, 3.0 $(NH_4)_2SO_4$, 1.49 $Na_2HPO_4$, $2H_2O$, 1.0 glutamate, 0.4122 $MgCl_2$, $6H_2O$, 0.0192 $ZnCl_2$, 0.0006 $CuCl_2$, $2H_2O$, 0.0044 $MnCl_2$, $4H_2O$, 0.0005 $CoCl_2$, $6H_2O$, 0.0174 $CaCl_2$, 0.0116 $FeCl_2$, $4H_2O$, 0.0003 $(NH_4)_6Mo_7O_24$, $4H_2O$, 0.0030 $H_3BO_3$ and a vitamin solution (g/L): 0.001 4-aminobenzoic acid, 0.125 myo-inositol, 0.005 nicotinic acid, 0.005 pantothenic acid, 0.005 pyridoxine, 0.005 thiamine HCl and 0.000012 biotin.

#### 2.1.3. Inoculum and bioreactor conditions

Five batch fermentations were carried out with similar operational and nutritional conditions. In each fermentation run, two cryovials of the mutant strain *X. dendrorhous* 25-2 were inoculated into a 125 mL flask with 25 mL containing culture media and incubated for 48 h, at a temperature of 20 °C and 250 rpm. Later 10% of the preculture was inoculated into a 500 mL flask containing 100 mL of the media under the same conditions for 48 h. Two shake flasks (1 L) with 200 mL of the culture media were inoculated with 10% of total volume from the previous culture under the same conditions for 36 h. The total volume of one of these two flasks was used to inoculate the bioreactor. Five batch fermentations were carried out in duplicate in 3 L Applikon® bioreactor sterilized at 121 °C for 15 min. The carbon source was sterilized by separate to avoid salt precipitation at 121 °C for 10 min. The fermenter was filled with 1.7 L of the culture media. The following physical variables were controlled: pH 5.5, temperature 20 °C, agitation speed 900 rpm and airflow 1 vvm. For monitoring and control purposes, a biocontroller Applikon ADI 1030 and the data acquisition software Bioexpert® were used. Samples were taken every 4, 6, 12 and 24 h during 144 h, depending on the fermentation time.

### 2.2. Analytical methods

#### 2.2.1. Cell growth, substrate and carotenoids quantification

Cell growth was determined using three different techniques: turbidity measured at 600 nm, counting in a Neubauer chamber and by dry weight. The reducing sugar concentration in the medium was measured using 3,5-dinitrosalicylic acid reagent [30], and the carotenoids concentrations by the DMSO method [31].

#### 2.2.2. Dielectric spectroscopy

A Fogale® nanotech dielectric spectroscopy probe attached to an EVO® Box was used to detect cellular viability on-line. The dielectric probe has a pair of electrodes that generate an alternating electric field in the culture media, the cells with healthy membranes behave like capacitors that can be polarized while lysed cell cannot. The electrical charge stored by the cell is expressed as the dielectric permittivity (pF/cm) which can be calculated by the Cole-Cole equation as:

$$\varepsilon(f) = \frac{\Delta\varepsilon\left(1+\left(\frac{f}{fc}\right)^{(1-\alpha)}\sin\left(\frac{2}{\pi}\alpha\right)\right)}{\left(1+\left(\frac{f}{fc}\right)^{(2-2\alpha)}+\left(\frac{f}{fc}\right)^{(1-\alpha)}\sin\left(\frac{2}{\pi}\alpha\right)\right)}+\varepsilon_\infty \quad (1)$$

where $(\varepsilon)$ is the permittivity as function of the frequency $f$, $\Delta\varepsilon$ is the increment between the low frequency and the residual permittivity, $fc$ is the characteristic frequency, $\alpha$ is a parameter which establish the slope of the permittivity curve and $\varepsilon_\infty$ is an offset value typically corresponding to the culture media without cells [6]. The permittivity increment can be expressed as:

$$\Delta\varepsilon = \frac{9PrCm}{4} \quad (2)$$

where $P$, $r$ and $Cm$ are the total cell volume fraction, the average cell radius and the capacitance of the cell membrane. Cellular viability $(\vartheta)$ is expressed as $\vartheta=\Delta\varepsilon\rho$, where $\rho$ is a correlation factor between $\Delta\varepsilon$ and the experimental biomass. Biomass concentration is calculated as the difference between two frequencies and the result is expressed as the absolute dielectric permittivity increment (pF\cm). The characteristic frequency (2 Mhz) was calculated with the software EVO® 400 assuming an average yeast size of 5 μm,