PHYSICAL METHODS APPLIED TO BIOTECHNOLOGY

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Contents

Summary

Suitable physical methods are critical for the separation and characterization of biological products. This applies to every bioprocess proposed to date but the complexity of this matter strongly depends upon the nature of the raw material and/or substrate used and the steps required for isolation and purification of the bioproduct(s). Since the objective of this article is beyond the presentation of each one of these methods, a specific and yet representative primary and secondary phytobiomass model (the juice and the bagasse of sugar cane) has been chosen to describe the analytical bottlenecks associated with biomass utilization. Many questions with regard to the application of each method have been raised in our discussions and both qualitative and quantitative assessments of the bioprocess under consideration have been addressed. Other simpler methodologies such as evaporation, lyophilization (freeze-drying), and crystallization have not been covered herein.

1. Introduction

Since Biotechnology deals with the utilization of life matter or specific biocatalysts (e.g.

enzymes) to generate goods and services, industrial plants and research laboratories participating in this kind of enterprise, or simply monitoring the timecourse of a scientific discovery for the consolidation of a new technology, make use of several physical methods of analysis. Thus, for the assessment of the various bioprocesses depicted in Figure 1, the most useful techniques employed for the analysis of substrates, biocatalysts (enzymes), and products are: spectroscopy, spectrometry, chromatography and, more recently, capillary electrophoresis as its micellar electrokinetic mode (Table 1). They may be employed both as complementary or confirmatory analytical tools. For instance, a routine chromatographic (thin-layer chromatography on silica-gel plates) or electrophoretic (polyacrylamide or agarose gel electrophoresis) procedure can be applied either to the simple qualitative and/or quantitative analysis of fermentation products such as secondary metabolites or to the sophisticated analysis of GMO (Genetic Modified Organisms) and TP (Transgenic Plants) genes through the numerous nucleic acid fragments obtained after the application of REFM (Restriction Enzyme Fragmentation Map) and/or PCR (Polymerase Chain Reaction).

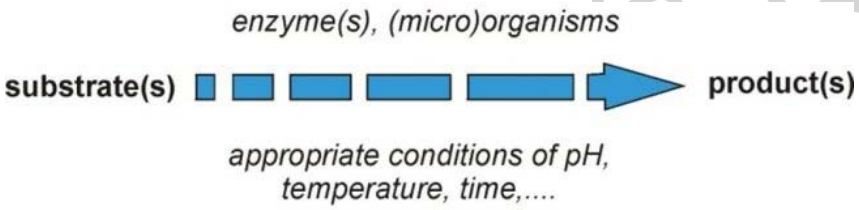

Figure 1. A simplified equation that rules biotechnological applications.

Acronym	Method	Application to the model
HPLC	Ion-exchange chromatography	Analysis of mono and oligosaccharides in steam-exploded bagasse hydrolysates
CGC	Capillary gas chromatography	Identification and quantitation of hemicellulose sugars in the form of alditol acetates
TLC	Thin-layer chromatography	Identification of microbial carotenoids in fermentation broths
DM	Densitometry	Quantitation of microbial carotenoids directly on a TLC plate
UV-vis	Spectrometry within the ultraviolet and visible spectral range	Characterization and quantitation of microbial carotenoids in fermentation broths
FTIR	Fourier-transformed infrared spectroscopy	Characterization of functional groups in steam-exploded bagasse
MCE	Micellar capillary electrophoresis	Characterization and quantitation of aromatic compounds (phenols) in bagasse hydrolysates
^{13}C-NMR	Nuclear magnetic resonance of ^{13}C	Characterization of polysaccharide (xylan) in sugar cane bagasse

Table 1. Physical methods of analysis applied in this study.

For the sake of a better understanding of the applications of the above mentioned techniques, several examples of contemporary biotechnology can be used to illustrate how these physical methods of analysis are useful to collectively monitor, analyze, and quantify all steps in the pretreatment, hydrolysis, fermentation and upgrading of natural goods such as sucrose (sugar cane juice and molasses), starch (from cereals like corn and wheat or from tubers like potato or cassava), cellulose and hemicellulose (sawdust from timber mills or husks from cereals) to their final products such as yeast biomass (MBP, Microbial Biomass Protein for animal feed), ethanol (fuel for more environmentally friendly vehicles), and oxygenated carotenoids (for fish farming and poultry). In this chapter, the bioconversion of sugar cane has been chosen as an example because this plant biomass provides both a soluble (sugar cane juice) and an insoluble (sugar cane bagasse) substrate for fermentation (Figure 2). Therefore, analytical problems related to both strategies can be adequately demonstrated and discussed.

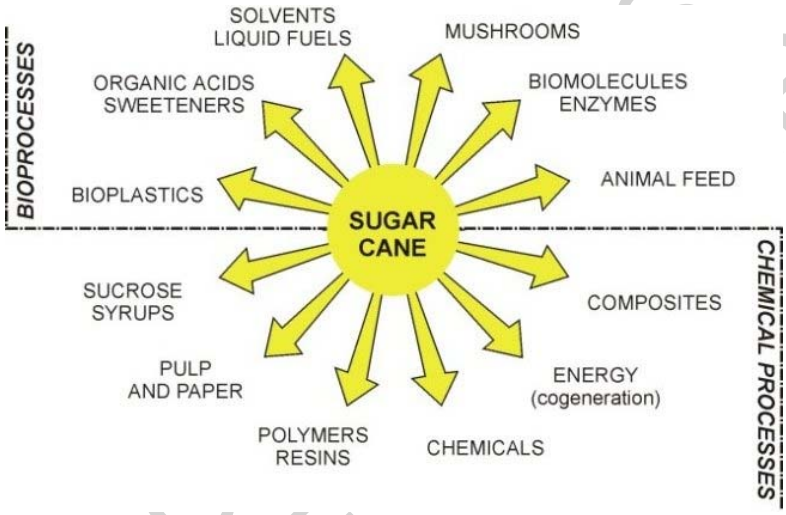

Figure 2: Utilization of sugarcane in chemical and biotechnological Processes

2. The Characterization of Potential Feedstocks in Sugar Cane

Lignocellulosic materials are inexhaustible resources that can be directly or indirectly used for the production of biomolecules and commodity chemicals. However, the industrial utilization of these renewable resources is compromised by several factors such as the close association that exists among the three main components of the plant cell wall (cellulose, hemicellulose and lignin) (Figure 3) and the low efficiency by which lignocellulosic substrates are converted through biological processes such as enzymatic hydrolysis and fermentation.

Sugar cane is one of the richest sources of carbohydrates in Nature and on a moisture-free basis it can provide 150kg of fermentable sugars (sucrose or table sugar) and 125kg of bagasse, a ternary complex of cellulose, lignin and hemicellulose (xylan) in a proportion of 45:20:35 in dry weight. More importantly, the cane juice contains approximately 20 percent of sucrose as a net result from photosynthesis, one of the highest performances amongst all plant materials.

For many decades, sugar cane plantations have been largely exploited as a reliable source for a number of commercial applications ranging from heat-generating materials (sugar cane bagasse) to renewable feedstocks for the chemical industry (e.g. sucrose, citric acid and polyhydroxyalcanoates). For instance, extensive plantations have been established in Brazil to fulfill the local demand for biofuels such as ethanol. Ethanol is widely recognized as the more ecologically satisfying fuel because of its advantages for the environment and its social and economical viabilities (see also - *Production of Alcohol for Fuel and Organic Solvents*). In Brazil, the sugar-ethanol market trade reaches about US$ 7.5 billions per year taking into consideration direct and indirect payments. This sector raises Brazil to the position of the largest world producer of sugar from sugar cane, and the sole country to implant a large scale, renewable, alternative fuel to petroleum. During the 1997/1998 harvest season (one year crop), more than 280 million metric tons of sugar-cane were produced, and from these 14.8 million metric tons of sugar and 13.8 billion liters of ethanol (5.41 billion liters in the form of anhydrous ethanol) were used for blending with gasoline, usually in a proportion of 22 to 24 percent of ethanol by volume.

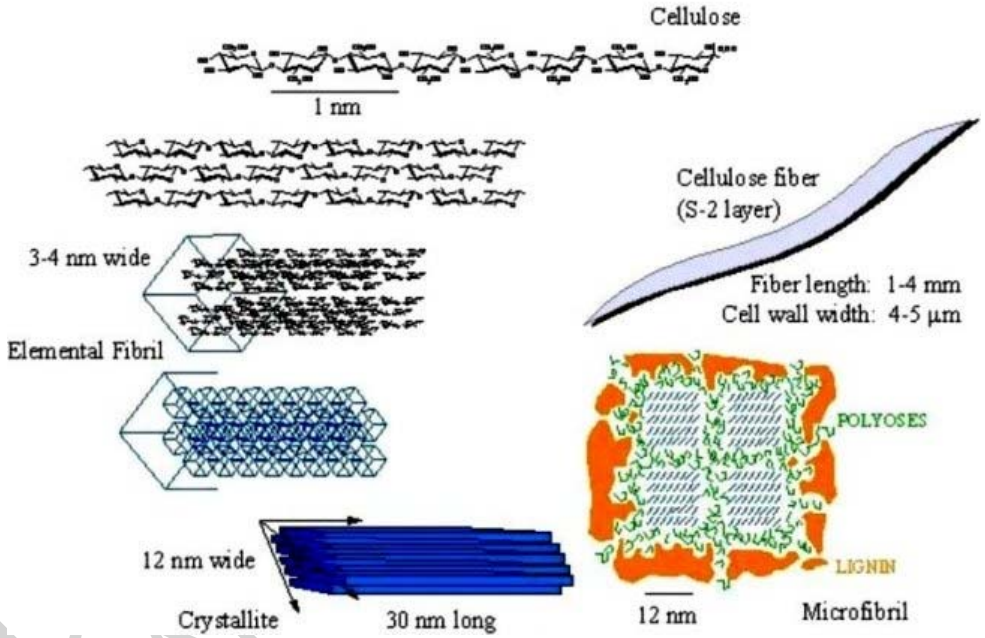

Figure 3**:** Supramolecular organization of cellulose and its association with lignin and hemicellulose in plant cell walls

Because of its high sucrose content, the juice extracted from sugar cane is considered one of the most readily available raw materials for bioconversion purposes, and many bioprocesses can be established on the basis of its availability. However, apart from its high availability and ease of conversion, the exploitation of sugar cane juice accumulates large amounts of bagasse with an almost negligible commercial value. Even though this process residue has been increasingly used for energy production such as in cogeneration, there is an enormous potential associated with the use of bagasse as a starting material for a wide range of applications (Figure 2).

At this point, it is important to emphasize that what we describe here for sugar cane also

applies to many other lignocellulosic feedstocks such as wheat straw, rice straw, corn stover, cassava bagasse, orange peel, wood sawdust, among others. For instance, eucalypt (*Eucalyptus* sp.) plantations, which now exist in Brazil, are the largest in the world and both bracatinga (*Mimosa scabrella*, a fast growing leguminous tree) and exotic pine (*Pinus taeda* and *P. elliottii*) plantations are also expanding. Depending on the regional climate and soil fertility, fast growing Southern-hemisphere hardwoods such as *Eucalyptus* can not only have growth rates of over 50 $m^3.ha^{-1}.year^{-1}$ but also display the ability to coppice from a recently cut stump. Therefore, considering the relatively large sustainability of reforestation programs based on these trees, there is a great opportunity for the utilization of their harvesting and processing residues as renewable sources for chemical feedstocks such as fermentable sugars, phenols, food additives, among others.

For the purpose of this article, the understanding of the nature of raw material used is an important step towards the recognition of its biotechnological potential. Without knowing their chemical composition and understanding their natural susceptibility to bioconversion, no one can possibly conceive a bioprocess that is feasible and economically viable. Many variables are involved and the quality of the chemical constitution of potential feedstocks may be critical for the process evaluation. Therefore, from this point on, we will focus on the methods used for a careful and integrated characterization of the feedstock and, for the examples precluded at the introduction of this chapter, we will describe the steps required for the generation of reliable experimental data which will, in turn, translate into technically viable bioprocesses.

3. Physical Methods used for the Characterization of Lignocellulosic Materials

Characterization of complex mixtures such as those described in this article requires a long and tedious analytical approach, sometimes involving both wet chemistry and several chromatographical and/or spectroscopical methods. This section describes the general characteristics of the most important physical analytical methods used to date in biotechnology, namely high-resolution liquid and gas chromatography, capillary electrophoresis, light-absorption spectroscopy and nuclear magnetic resonance. Examples are provided in relation to the application of each of these methods in the bioprocessing of our phytobiomass of choice, sugar cane.

3.1. Light-absorption Spectroscopy

In general, the term spectroscopy represents the investigation of interactions that exist between matter and an incident radiation of known wavelength (λ: gr., *lambda*), and the graphic relationship that describes these interactions is called spectrum. A selected range of wavelengths from the electromagnetic spectrum will then dictate whether the analytical method is either, ultraviolet (190-340 nm), visible (340-750 nm) or infrared (>850 nm or 400 to 4000 cm^{-1} for the mid-infrared and 2000 to 10000 cm^{-1}fom the near-infrared region) spectroscopy. On the other hand, the term spectrometry (or spectrophotometry) is applied to measurements that are carried out on a given spectrum for the calculation of the concentration of an analyte or functional group of related molecules. Therefore, while spectroscopy is a strictly qualitative method (different species will absorb at different wavelengths), spectrometry provides quantitation of

light-absorbing species according to Beer's Law. In short, given the experimental limits, the amount of light absorbed (absorbance, A) is directly proportional to the sample concentration, to the light path within the sample (cell width) and to a constant that characterizes the sample specimen or analyte (absortivity, ε) *(see also - Microbial Cell Culture)*

Ultraviolet-visible spectroscopy (UV-vis) has been widely used for the characterization of chemicals in solution but its application is usually attached to a chemical derivatization method in which chromogenic substances are generated. A selective application of this method for the characterization of microbial carotenoids is given below (*see also - Chemical Methods Applied to Biotechnology*).

The application of Fourier-Transformed Infrared Spectrocopy (FTIR) to lignocellulosics has been increasingly pursued as a fast and direct method for the characterization of functional groups in phytobiomass. In this case, both transmission and diffuse reflectance modes of FTIR can be applied to fibers and milled samples, aiming at characterizing the relative chemical composition of these materials and trends involved in pretreating and bioconverting lignocellulosics. The difference, however, between these two modes is that the former requires sample milling and its incorporation into almost translucid potassium bromide (KBr) disks (typical sample concentration of 1 percent in relation to the mass of anhydrous KBr), whereas the latter allows for the direct measurement of the sample but, as a result of the heterogeneity of the fibrous material, usually renders FTIR spectra more scattered than those obtained by transmission FTIR.

The infrared spectrum is generated as a result of the exposure of a test sample to specific wavelengths within 700 to 5000 nm, usually expressed as their corresponding wave numbers (cm^{-1}). Infrared absorption results from changes in the vibrational and rotational state of a molecular bond (e.g. C-C and C-H bonds in alkanes, C=O in carbonyls of aldehydes and ketones, C-O and O-H bonds in alcohols, C-H and C-C bonds in aromatic rings, C-O-C bonds in ethers, etc.) as a result of coupling with the incident eletromagnetic radiation. Therefore, absorption can only occur if the vibrating molecule produces an oscillating dipole moment that can interact with the electric field of the radiation. This way, each chemical bond can be identified by absorption bands that are located at several specific wave numbers.

A good FTIR spectrum usually gives plenty of information. The interpretation of this is usually complex due to peak overlap and broadening. Therefore, the attribution of specific shifts on FTIR bands to changes in chemical composition is not usually an easy task. However, through the application of multivariate analysis of the FTIR data, a suitable simplification of the spectrum is obtained and changes in chemical composition and structure are more easily observed. This technique has largely been applied to lignocellulosics, as indicated in several recent publications in this field. In this way, almost superimposed FTIR spectra can be grouped in cartesian coordinates by principal component analysis, in which the two first principal components are used to explain over 90 percent of spectral variations that are not easily observed by the naked eye.

The Raman light absorbing spectroscopy has been increasingly used for the non-

destructive characterization of phytobiomass. Similar to FTIR, Raman spectroscopy is used to determine molecular structures and composition of organic and inorganic materials. Sample preparation for Raman spectroscopy is much simpler than that required for infrared spectroscopy but the Raman scattering is a relatively inefficient process, somewhat 10^3 fold weaker than the Rayleigh scattering used in FTIR. Therefore, very intense excitation sources are required such as a laser beam.

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Biographical Sketches

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Nadia Krieger is an adjunct professor in the Department of Chemistry at the Federal University of Parana (UFPR), Brazil. She graduated in chemistry and did her PhD in biochemistry in a sandwich program between the Department of Biochemistry at UFPR and the Centre for Biological and Chemical Engineering, Superior Technical Institute (Instituto Superior Técnico), in Lisbon, Portugal. She works in

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Luiz Pereira Ramos, Researcher Level 2A of the National Research Council for Scientific and Technological Development of Brazil (CNPq), belongs to the permanent staff of the Federal University of Paran (UFPR), Department of Chemistry since 1986 and presently has the status of an Adjunct Professor. His B.Sc. in chemistry (1982) was obtained at the Catholic University of Parana, whereas his M.Sc. in biochemistry (1987) was obtained at the Department of Biochemistry of UFPR. His Ph.D. in biology (1992) was concluded at the Department of Biology of the Ottawa-Carletton University, Ottawa, Ontario, Canada and his post-doctoral venue (1996) was carried out at the University of British Columbia, Vancouver, Canada. As a result of his contributions on the upgrading of agricultural byproducts, he was awarded with the "Professor Antenor da Silva Puppo" Prize in Biotechnology from the Regional Council of Chemistry (1994).