CHEMICAL METHODS APPLIED TO BIOTECNOLOGY

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Keywords: chemical methods, biotechnology, proteins, carbohydrates, lipids, nucleic acids

Contents

Summary

Chemical methods have been, are, and will be a mandatory set of the techniques for the characterization and quantification of promptly fermentable feed stocks, for the pretreatment of polymeric substrates and also for similar purposes when dealing with the downstream processing of fermentation goods. Chemistry (even when re-qualified as biochemistry) as well as physics and applied microbiology are still the main knowledgeable areas supporting most of the current biotechnological processes,

meanwhile applied genetics and genetic engineering make room in the revolution of modern biotechnology**.**

1. Introduction

Analytical methods are used or at least are useful in all applications of biotechnology. However, in the space available, it is impossible to give details of all methods. Therefore, in keeping with the overall perspective, the focus will be where analytical methods are used to determine concentrations in order to monitor the progress of fermentation or to determine purity and composition of a sample in order to evaluate the success of recovery and purification steps in the subsequent downstream processes.

Analytical methods can be divided into chemical and physical analytical methods. However, there is not a distinct boundary between both types of methods. For example, an analysis may involve a chemical reaction to generate a colored substance, followed by the use of a spectrophotometer, which functions according to purely physical principles, to measure the concentration of the colored substance. The approach taken here is to discuss where chemical reactions are used either in sample preparation or in the analysis itself. In addition, many physical methods will be mentioned in this section to facilitate comparisons when both physical and chemical methods are available. An important example is a general trend within biotechnology to substitute many of the chemical analyses described below by high-pressure liquid chromatography (HPLC) methods. This equipment is quite reliable and the analysis quite easy and fast to perform, as long as the equipment has an automatic injector. The drawback of this technique is the need of comparison to reference products, for the strength of the technique lies in its ability to separate the compounds within a sample; it does not in itself identify the compounds. Injecting known compounds this identification can only be performed by injecting known compounds into the HPLC and comparing how these behave compared to the unknown compounds in the sample. Standards for HPLC can be quite expensive or they may even not exist in the case of a new metabolite (see also *Physical Methods Applied to Biotechnology*]

This article is organized according to the main divisions used to classify the substances present in living matter: carbohydrates, proteins, lipids and nucleic acids. Figure 1 shows the role of these various methods in the analysis of fermentation and downstream processes. Note that the process could be a routine production process, or alternatively it could form part of a research and development scheme for a new process. The methods used will depend on what features of the system need to be characterized, which in turn depends on the objective of the work. For example, to understand how product formation is related to growth, it will be necessary to take samples at various times during the fermentation, and to use appropriate methods to determine the biomass, substrate and product concentrations. The information gained from sample removal at different times may allow specific growth, substrate consumption and product formation rates to be calculated. It may be possible to go even further and propose kinetic equations to characterize the system. Combining these equations to develop a model for the system (see also *Mathematical Modeling in Biotechnology*), which will summarize process performance may be a useful tool to guide process optimization (see also *Process Optimization Strategies for Biotechnology Products*].

2. Methods for Protein Determination

A protein is a polypeptide consisting of a linear chain of amino acid residues, these residues being linked to each other by peptide bonds. The sequence of the amino acid residues in the chain is referred to as the primary structure of the protein. Since proteins contain many amino acid residues (from a few tenths, for example, in insulin, to several hundreds, for example, in phosphorylase, resulting in molecular weights of respectively 5700 to 370 000 Da or more), the linear chain is able to fold and assume complex three-dimensional shapes, various aspects of which are referred to as secondary, tertiary and quaternary structure. The three-dimensional structure is maintained through a large number of non-covalent interactions between amino acid side chains and between atoms within peptide bonds. These interactions include hydrogen bonds, hydrophobic interactions and Van der Waals forces. In some proteins covalent disulphide bonds are formed between the side chains of two residues of cysteine located in distant regions of the molecule, conferring a significant degree of stability to the three-dimensional structure.

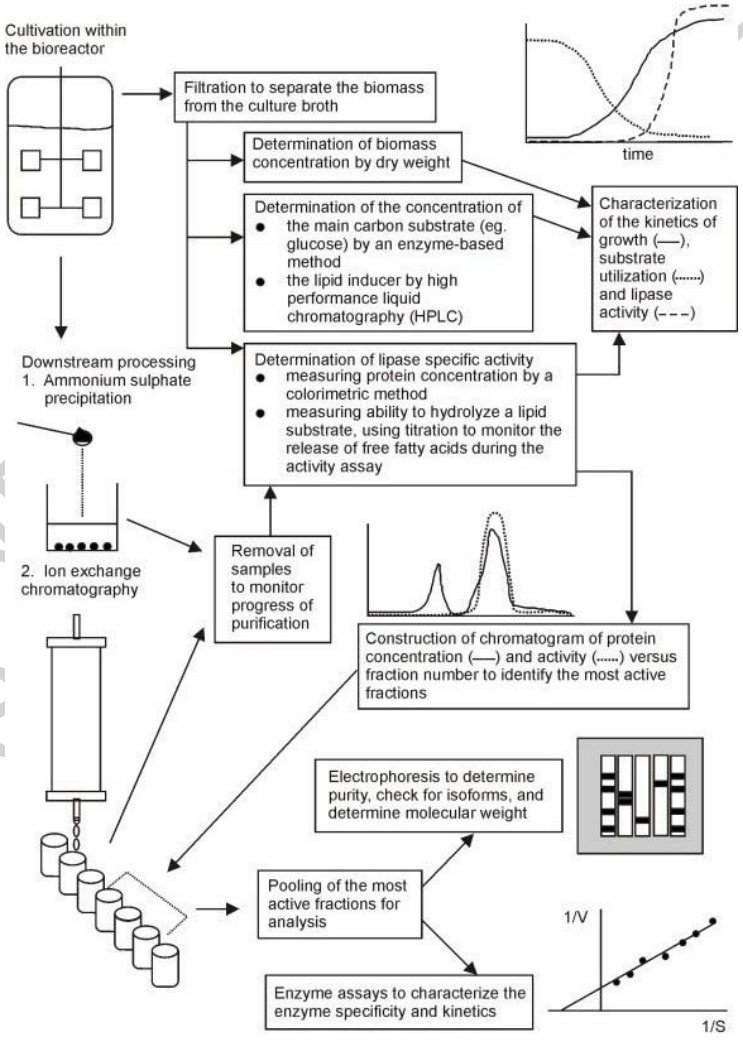

Figure 1: A flow sheet depicting a biotechnological process on the production of a commercial lipase

In biotechnology it is frequently necessary to determine the quantity of protein present in a sample, for example for:

- The use of protein to indicate the amount of microbial biomass in a sample. Typically there are not large variations in the protein content of microbial biomass during the growth cycle. The protein content of the biomass can be determined in separate experiments and then protein measurements can be used to follow the kinetics of microbial growth during fermentation. Typical protein contents are 60 to 65 percent of the dry cell weight for bacteria, 45-55 percent for yeasts and 40 to 50 percent for fungi. However, this method cannot be used if there are non-cellular or water insoluble proteins in the medium components, since it is based on the measurement of insoluble protein.
- The determination of the specific activity of enzymes during their production and purification. The enzyme activity in a sample is divided by the protein content measured in the sample to calculate the specific activity. This quantity increases as the purity of the enzyme increases and therefore is a good indicator of the success of the purification scheme.

Protein determination methods have been widely investigated and discussed over the last 50 years. The most commonly used methods are colorimetric or chromogenic: the Biuret method, the Lowry method, Coomassie Blue (CB)G-250 dye binding (Bradford assay) and the bicinchoninic acid (BCA) assay. The Kjeldhal nitrogen procedure is often cited as a reliable method to determine the "exact" concentration of the protein. However, this method is time-consuming and reagent-consuming, and therefore is not frequently used in research laboratories. On the other hand, it has been increasingly used in the biopharmaceutical industry.

The majority of these methods depend not only on the quantity of protein but also on their composition. Different proteins contain different proportions of the 20 major amino acids and some also contain covalently bound molecules, such as carbohydrates. The protein conformation will affect which reactive groups are available to react with the reagents used in the assay. It is worth noting that no method gives a fully accurate measurement, unless it has been calibrated against a protein solution of identical composition. In practice, this is not an easy task because the pure protein is required to perform the calibration curve. Normally, pure bovine serum albumin (BSA) is used to standardize a method and therefore, one must be aware of the possibility of having an intrinsic error in the measurement of protein in the sample. Care must be taken to ensure that the standard curve remains linear over the range of the protein concentration tested.

Table 1 gives the main characteristics of the chemical methods most commonly used in protein determination. It is clear that most of the current colorimetric methods for the determination of protein concentration depend on the protein composition. In addition, many of them have several interfering substances, although the effects of these can often be minimized by precipitating the protein prior to the assay or even by diluting the sample, as many methods are of high sensitivity. The selection of an appropriate standard together with a rigorous validation of the process can solve the problem of the protein composition.

Method	Principle	Sensitivity	Advantages	Comments	Main Interfering compounds	Time required	Amount of protein needed (mg)
Kjeldhal	Acid-base titration of the total nitrogen of the sample	low	Reliability	Time and sample consuming	Non-protein nitrogen	several hours	
UV absorption (280 nm)	Tyrosine and Tryptophan residues absorb at 280 nm	0.2-2 mg/mL	Direct, rapid, nondestructive	Dependent on the protein composition (Tryptophan, Tyrosine and phenylalanine)	Nucleic acids and UV absorbing materials	Instantaneous	0.05-2
Biuret	Purplish-violet copper-protein complex	low	Independent of the protein composition	The variation in colour from protein to protein is low. Copper reagent reacts with the peptide chain.	Tris buffer, ammonium ions, sucrose, primary amines and glycerol.	Fast (5 min)	0.05-5
Lowry	Dark-blue copper-protein complex	5-100 µg/mL	High Sensitivity	Dependent on the protein composition due to involvement of the residues of tyrosine and tryptophan in the complex formation	Nitrogen containing buffers, phosphate salts, ammonium ions, detergents, sugars, chelators, reducing agents	40 min.	0.05-5
Bradford assay	Blue dye (Comassie-blue G-250) protein complex	25-200 µg/mL	Rapidity and high sensitivity. Few interfering substances.	High dependence on the protein composition	Some detergents (SDS, Triton X-100)	10 min.	0.01-0.05
Bicinchoninic acid (BCA) assay	Variation of the Lowry method: BCA-Cu^{+1} complex	Standard protocol: 20-2000 µg/mL Enhanced protocol: 5-250 µg/mL	Single reagent. End product is stable. Fewer interfering substances than Lowry assay	Dependent on the protein composition	EDTA, glucose, reducing agents, ammonium ions	40 min., 2 h or overnight	0.02-0.12 (standard protocol) 0.005-0.025 (enhanced protocol)

Table1: Principal methods used in protein determination

Some recent methods of protein quantification seem to overcome the limitation presented by the classical techniques showed above. These methods use fluorogenic reagents, such as fluorescamine, *o*-phthaldialdehyde (OPA) or 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) which binds to primary aliphatic amines of amino acids, peptides, and proteins, or to protein-lipid complexes by hydrophobic interactions. These are quite sensitive methods that can detect protein quantities in the range of 10 to 1000 ng.

Antibody based methods can be used for the determination of specific proteins within protein mixtures. Radioimmunoassays are widely used to detect small amounts of a specific protein and other biological substances in both clinical and biotechnological applications. Likewise, the ELISA method (enzyme-linked immunosorbent assay) involves the binding of a specific protein to an antibody. The amount of protein is determined by adding a second antibody to which is bound an enzyme that catalyzes a chromogenic reaction.

Evidently, in spite of the fact that proteins play crucial roles in nearly all biological processes, their quantification is not a simple task. Many efforts have been made to find a reliable and sensitive method that is convenient to use at every stage during recovery of proteins from the fermentation broth and the subsequent purification steps. Most methods of analyzing and purifying proteins are based on chromatography, mainly electrophoresis, ion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography and affinity chromatography. Most of the devices can be operated either in preparative mode to purify the protein, or in analytical mode to separate and quantify the various components of the sample (see also *Downstream Processing of Proteins using Foam Fractionation*]. Once a protein has been isolated and purified so that it is free of contaminating substances, various methods can be used to determine specific characteristics of the protein.

2.1. Determination of Protein Mass and Structure

Consider a typical biotechnological process, the goal of which is to search for an enzyme with new properties or a new enzyme (see also *Enzyme Production*). One must investigate: a) the production of the enzyme: the producing microorganism, the optimal conditions for production, the location of production (intracellular or extracellular), the folding state and the presence of other enzymes or macromolecules which may interfere in the whole process; b) the kinetic characteristics of the enzyme, the temperature and pH ranges for optimal activity, the stability, the aggregation tendency and the need for cofactors; and c) the physical parameters of the enzyme such as multimeric state, molecular weight and charge (pI, isoelectric point).

Prior to starting these complex processes, it is essential to collect as much information about the target protein as possible. Knowledge of the characteristics of the enzyme may justify the efforts to develop a new production and purification process by demonstrating the commercial potential and may also allow the development of strategies for isolation from the fermentation broth and further purification. Similar considerations would also apply in the development of a process for the production of a protein with therapeutic properties.

The determination of the protein mass and structure requires an array of methods, which are based on physical-chemical characteristics of the protein, namely size, solubility, charge, and binding affinity. Many of the methods required for these analyses are beyond the scope of this chapter, which focuses on chemical methods. Nevertheless, it is worthwhile to mention the most important methods here, for a more comprehensive understanding of the whole process of production, isolation and characterization of a protein. These include: electrophoretic methods; circular dichroism (CD), which is used to determine the protein secondary structure; analytical ultracentrifugation, which determines the sample homogeneity, the interactions with other molecules, and the interactions inside the required protein molecule; mass spectrometry (MS), which allows the determination of the protein mass with a remarkable precision; Fourier transform infrared spectroscopy (Ft-Ir), which can be used to evaluate structural characteristics of proteins, including secondary structures, protein dynamics and thermal denaturation; and nuclear magnetic resonance (NMR), which can be used to determine protein structure and protein-ligand binding and protein mobility.

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Biographical Sketches

Nadia Krieger, Researcher Level 2 of the National Research Council for Scientific and Technological Development of Brazil (CNPq), is an adjunct professor in the Department of Chemistry at the Federal University of Paraná (UFPR), Brazil. She graduated in chemistry , did her M.Sc. in Food Technology at the Engineering Department of UFPR (1987), and her PhD in biochemistry (1995) 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. Her post-doctoral venue (2001) was carried out at the "Universitée de la Mediterranée", Marseille, France. She works in the production (either by submerged fermentation or solid state fermentation), purification, characterization and application of enzymes (biocatalysis).

Luiz Pereira Ramos, Researcher Level 1 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 Paraná, 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-Carleton 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).

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