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SHORT CONTRIBUTION

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A simplification of the protein assay method of Ramsay et al. for the quantification of *Thiobacillus ferrooxidans* in the presence of ferric precipitates

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Abstract A variation on Ramsay's method for microbial protein determination has been developed in order to quantify *Thiobacillus ferrooxidans* attached to ferric precipitates or in aqueous suspensions containing such precipitates. Some modifications have been introduced to provide a method that is more sensitive, simple and rapid. A linear standard curve is presented to permit a direct correlation between the protein concentration (mg/l) and the cell concentration (10^6 cells/ml). An application of this method has been demonstrated in the quantification of biomass immobilized on the surface of polyurethane foam particles in a packed bed reactor, several experiments having been conducted to establish the best conditions for the quantification studies.

Introduction

Thiobacillus ferrooxidans is a chemolithotrophic bacterium that is able to oxidize ferrous iron in acid solutions, coupling the energy thus derived to support carbon dioxide fixation and growth. The bacterial oxidation of ferrous iron is based on the following reaction:

$$2FeSO_4(aq) + H_2SO_4(aq) + 1/2O_2 \xrightarrow{\textit{Thiobacillus ferrooxidans}} Fe_2(SO_4)_3(aq) + H_2O$$

This ability is suited to the bioleaching of minerals and the treatment of acid mine drainage. In addition, *T. ferrooxidans* has been used for treating "sour" gases containing H_2S. This application, though very attractive compared with chemical alternatives because of its lower environmental impact and for economical reasons, has not yet been widely used commercially (Jensen and Webb 1995a, b).

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The natural tendency of *T. ferrooxidans* to grow on surfaces makes it an ideal microorganism for cell immobilization and a number of workers have exploited this tendency in attempts to increase ferrous iron-oxidation rates (Grishin and Tuovinen 1988; Karamanev and Nikolov 1988; Halfmeier et al. 1993; Armentia and Webb 1992; Jensen and Webb 1994; Nemati and Webb 1996, 1997, 1998, 1999). As a consequence, cell concentration and distribution on support matrices are essential parameters for investigating the performance of the bioreactor. In this sense, it is necessary to provide a rigorous and accurate method for quantification of free cells and immobilized cells.

It has been observed that during ferrous iron oxidation by *T. ferrooxidans*, large quantities of ferric precipitates are produced which cover the support particles that play a substantial role in the biofilm formation. These compounds cause significant interference in the estimation of the cell concentration.

Ramsay et al. (1988) proposed a modification of Peterson's method (Peterson 1977) for microbial protein determination with the aim of removing interference from solid precipitates. They developed a method in which oxalic acid was used to remove ferric precipitates and release the cells trapped within precipitates. To estimate the total protein, after the addition of sodium deoxycholate-TCA (DOC-TCA) for rapid quantitative recovery of soluble and membrane proteins, the mixture was exposed to NaOH and high temperature to provide adequate solubilization of proteins and to precipitate residual ferric compounds. However, the alkaline treatment leads to a loss of sensitivity, with the highest absorbance values being obtained at a concentration of 400 mg/l.

In the study described here we have examined this method, confirmed and extended the original findings, and modified it where necessary in order to increase its sensitivity. The result is a simpler and more rapid procedure that provides an objective and reproducible estimation of cell concentrations.

Materials and methods

Microorganism and medium

The strain of *T. ferrooxidans* (UCA 2) used in this study was isolated in the Riotinto mines in Huelva (Spain) and was kindly made available by the Biohydrometallurgy Group of the University of Seville (Spain).

T. ferrooxidans was grown in a mineral medium containing (per litre demineralized water): $FeSO_4 \cdot 7H_2O$ (5 g); $(NH_4)_2SO_4$ (3 g); $MgSO_4$ (0.5 g); K_2HPO_4 (0.5 g); KCl (0.1 g); $Ca(NO_3)_2$ (0.01 g); $ZnSO_4 \cdot 7H_2O$ (5 mg); $CuSO_4 \cdot 5H_2O$ (0.5 mg); $MnSO_4 \cdot 4H_2O$ (0.5 mg); $CoSO_4 \cdot 7H_2O$ (0.5 mg); $Cr_2(SO_4)_3 \cdot 15H_2O$ (0.25 mg); $Na_2B_4O_7 \cdot 10H_2O$ (0.25 mg); $NaMoO_4 \cdot 2H_2O$ (0.25 mg); $NaVO_3$ (0.05 mg). The medium was adjusted to pH 1.6 with 5 M H_2SO_4 and sterilized by filtration.

Free cell concentration by direct counts

The cell concentration in free suspension was determined by direct microscopic counting using a Neubauer chamber of 0.1 mm depth and 1/400 mm^2 area.

Biomass support particles

Polyurethane foam cubes 1 cm long, having a density of 20 kg/m^3 and a porosity of around 96%, were employed as carriers for immobilization of *T. ferrooxidans* cells.

Immobilization procedure

Batch cultures for the immobilization of cells were performed in 500-ml Erlenmeyer flasks containing 200 ml mineral medium and 65 biomass support particles. The media were inoculated with 10% (v/v) cell suspension obtained from a culture in exponential growth and incubated on a rotary shaker for 48 h at 30 °C. Before complete consumption of the substrate (ferrous iron), the spent medium was replaced by fresh medium. Following this procedure, four consecutive batches were run without inoculation.

Protein assay method

The working solutions were as follows: 0.15% sodium deoxycholate, 72% trichloroacetic acid, modified Lowry reagent, Folin & Ciocalteu's phenol reagent, bovine serum albumin (0.4 mg/ml) (Sigma) and 10% (w/v) oxalic acid.

Preparation of cell-free extracts from cell suspensions

1. Centrifuge 6-ml culture broth at 12,000*g* for 20 min to harvest cells. If sample is too dilute, multiples of this proportion can be used to augment the total protein recovered in the precipitate.
2. Blot away the supernatant and add 1 ml oxalic acid to dissolve the ferric precipitates. Shake for 24 h at room temperature. The length of exposure to acid and the volume used depend on the amount of precipitates. The conditions outlined here are appropriate in the cultures used.

Preparation of cell-free extracts from immobilized cells

1. Take a number of biomass support particles and add 60% of the total volume of oxalic acid. The number of support particles and the volume of oxalic acid must be appropriate to release the cells and dissolve the ferric precipitates. Shake at 200 rpm for 24 h.
2. Discard the supernatant and add 25% of the total volume of oxalic acid to the support particles. Shake at 200 rpm for 2 h.
3. Discard the supernatant and wash the support particles with the remainder of the oxalic acid (15%), mixing well with the fractions obtained in the previous steps.

Protein determination

1. Take 1 ml free cell extract and add 0.1 ml 0.15% DOC. Mix well and allow to stand at room temperature for 10 min.
2. Add 0.1 ml 72% TCA, mix and centrifuge at 12,000*g* for 10 min. Remove the supernatant immediately by aspiration.
3. Add to the pellets, containing between 10 μg and 200 μg protein, 1 ml oxalic acid and 0.1 ml TCA. Centrifuge the solutions at 12,000*g* for 10 min, discarding the supernatant after centrifugation.
4. Dissolve the pellets in 1 ml Lowry reagent solution and 1 ml distilled water, mix, and allow to stand for 20 min at room temperature. Solubilization of the protein pellets is done immediately after the addition of Lowry reagent.
5. Add 0.5 ml Folin & Ciocalteu's phenol reagent and mix immediately. After 30 min, read the absorbance at 750 nm.

Results

Comparison with the methods of Peterson (1977) and Ramsay et al. (1988)

The method of protein analysis described above was compared with the method of Peterson and with the method of Ramsay et al. for several different protein standard solutions.

These standards were prepared by diluting protein solutions of 400 mg/l bovine serum albumin (BSA). The concentrations were varied between 12 mg/l and 100 mg/l. The dilutions were prepared with distilled water for the samples analysed by Peterson's method and with oxalic acid for the samples analysed by the method of Ramsay et al. and by the proposed procedure. Figure 1 shows the standard curve for Peterson's method and the standard curve for the proposed method.

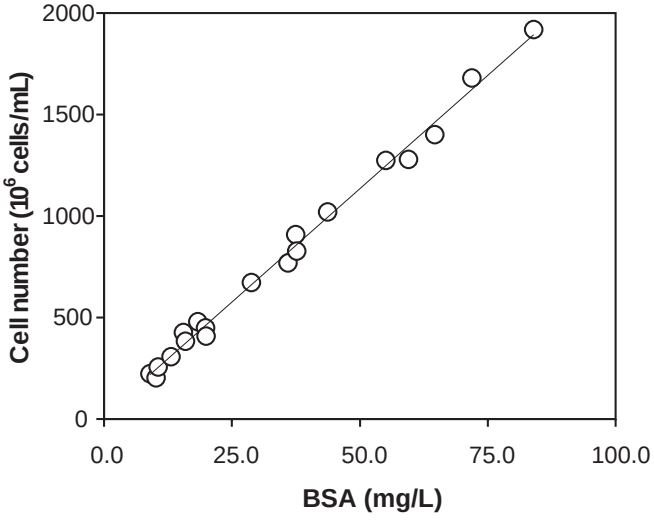

Fig. 1 Comparison between the Peterson method and the method described in this paper

The method of least-squares analysis for the linear region produced confidence intervals of ± 0.52 mg/l.

The effect of oxalic acid

In order to determine the effect of exposure of protein to oxalic acid over time, a number of experiments were performed in which the protein concentrations at various times were measured. The results showed that a time of exposure to oxalic acid between 24 h and 72 h did not significantly affect the protein concentration.

Correlation between total protein concentration and cell concentration

The bacterial concentration was determined in several submerged cultures (in exponential growth) by direct counting and the protein concentration was determined by the proposed method.

A plot of the bacterial concentration versus the protein concentration is shown in Fig. 2. Application of the method of least-squares analysis produced confidence intervals of $\pm 4.8 \times 10^6$ cells/ml. The use of this linear transformation provides, in direct form, the cell concentration from the measurement of absorbance values.

Measurement of biomass immobilized on the surface of polyurethane foam particles

In order to determine the immobilized cell concentration it was necessary to establish the number of support particles and the volume of oxalic acid necessary to release the cells completely and dissolve ferric precipitates. For this reason we performed several experiments with support particles from a culture prepared as described in the Materials and methods section. The rates used were as follows (support particles/volume of oxalic acid, in ml): 10/100; 10/200; 10/300; 20/100; 20/200; 20/300; 30/100; 30/200; 30/300. The protein assay produced an average result of $15 \times 10^9 \pm 3.4\%$ cells/g support. This suggested that the ratio between support particles and volume of oxalic acid does not significantly affect the determination of biomass.

Discussion

The results obtained using Peterson method and using the proposed method, for various protein standards, are similar. Nevertheless, the absorbances obtained using the proposed method were slightly lower.

Table 1 shows the results of the comparison between the method of Ramsay et al. and that proposed in this paper for different standards. It can be seen that the modification proposed in this paper leads to a significant improvement in the sensitivity of method and allows more dilute solutions of proteins to be analysed.

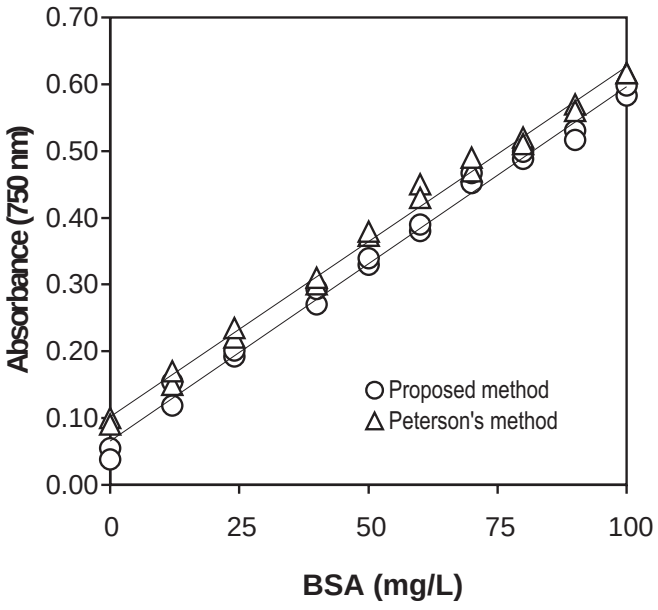

Fig. 2 Correlation between total protein concentration and cell concentration

Table 1 Comparison of the Ramsay method to that of this paper

Bovine serum albumin (mg/l)	Absorbance (750 nm)	
	Proposed method	Ramsay et al. method
0	0.03	0.00
25	0.20	0.02
50	0.32	0.05
100	0.60	0.12
400	–	0.47

In relation to measurement of biomass immobilized on the surface of polyurethane foam particles, it is possible to select of the most appropriate proportion for the assay, depending on the degree of colonization of the support.

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