Utilisation of an electro-optical method to investigate the amidase activity of microbial cells

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Abstract

The electro-optical (EO) properties of a microbial cell suspension of Brevibacterium sp. strain 13 PA were examined during acrylamide and acrylic acid metabolism and during culture of cells in a mineral medium without added carbon sources. The dependence of the suspension turbidity changes due to cellular orientation on the frequency of an orienting electric field (orientational spectra, OSs) over the range of 10–10000 kHz were used. Alterations in the OSs, depending on cellular amidase activity, were recorded during growth with acrylamide as the sole source of carbon. However, no amidase activity was observed in cells growing with acrylic acid as the sole carbon source. Growth on the carbon-free mineral medium resulted in a reduction in amidase activity. It is apparent that the OS changes reflect the corresponding changes in cell polarisability, which may be associated with acrylamide hydrolysis. The data generated from these studies can be taken as a basis for the EO determination of the enzyme activity of microbial biocatalysts. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: Enzyme activity; Electro-optical property; Brevibacterium sp.

1. Introduction

The measurement of the enzyme activity of microbial cells is frequently performed analogously to that of an individual enzyme. Conventional enzyme assays are normally based on the fact that in the course of the reaction there occurs a reduction or increase in the amount of substrate or product formed.

In this study, we sought to use an electro-optical (EO) method of analysing cell suspensions to assess the enzyme activity of native microbial cells. The EO analysis has been known [1,2] to permit, with reasonable effectiveness, keeping track of changes in cellular electro-physical characteristics that occur at the level of this or other structural components. The approach taken is based on the belief that cellular enzymic processes are attended with conformational and structural changes in the biomacromolecules

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that, in turn, can lead to adequate changes in the cellular electro-physical characteristics.

The attraction of EO analysis for monitoring microbial amidase activity was first reported in a previous paper [3], as were the basic principles of EO analysis and its applicability for studying enzymic processes in microbial suspensions. The aim of this study was to explore the effect of microbial amidase activity on the EO properties of cells during batch culture on various substrates.

2. Materials and methods

2.1. Microorganism

Brevibacterium sp. strain 13 PA, obtained from the Laboratory of Microbiological Transformation of the ‘Biocatalysis’ Science Research Institute (Saratov), was used. Cells of this strain possess an inducible enzyme, amidase, and can grow with acrylamide and/or acrylic acid as sole carbon sources.

2.2. Growth conditions

Fresh 24-h culture of strain 13 PA, grown on beef-extract agar (BEA) at 29°C, was transferred to a solid medium that contained: BEA, 15 ml; acrylamide, 5 g l\(^{-1}\); pH 7.5. After being grown at 29°C, the 24-h culture at the solid medium was washed off with a phosphate buffer (0.01 M K\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\); pH 7.6) and inoculated into 300 ml conical flasks containing the following: (1) 50 ml phosphate buffer, 0.232 g l\(^{-1}\) MgSO\(_4\), 0.392 g l\(^{-1}\) EDTA, and 2.8 g l\(^{-1}\) acrylamide; (2) 50 ml phosphate buffer, 0.232 g l\(^{-1}\) MgSO\(_4\), 0.392 g l\(^{-1}\) EDTA, and 5 g l\(^{-1}\) acrylic acid; (3) 50 ml phosphate buffer, 0.232 g l\(^{-1}\) MgSO\(_4\), and 0.392 g l\(^{-1}\) EDTA. Growth was conducted on a rotary shaker (160 rpm) for 48 h at 29°C.

2.3. Measurements of cellular orientational spectra

The orientational spectra (OSs) of the cells were determined with an ELBIC EO analyser at a light wavelength of 670 nm relative to vacuum. The analysis conditions were: volume of the measuring chamber, 1 ml; cell concentration (in optical density units) \(\text{OD}_{670} = 0.40-0.44\). Prior to use in the analysis, the cells were sedimented by centrifugation at 5000 \(\times\) \(g\) for 5 min, resuspended in deionised water and again centrifuged under the same conditions. The pellet which resulted was resuspended to an optical density (OD\(_{670}\)) of 0.5. To remove cellular aggregates, the cell suspension was subjected to a further centrifugation at 1000 \(\times\) \(g\) for 1 min, and the suspension that remained in the supernatant liquid was studied further. The cells thus prepared were incubated with 0.5 g l\(^{-1}\) acrylamide for 30 min at 35°C and subjected to EO measurements. A discrete set of frequencies of the orienting electric field (10, 52, 104, 502, 1000, 5020, and 10 000 kHz) was used.

An OS was presented as a frequency dependence of the difference of the suspension optical density values \(\Delta\text{OD}\) measured during the propagation of a beam of non-polarised light along and across the orienting field direction. This difference was normalised to the value of optical density measured for randomly oriented cells. There is reason to assume that the general view of the OS under the selected experimental conditions (light wavelength, amplitude of the orienting electric field strength, etc.) is essentially determined by the frequency dependence of the anisotropy of the cell polarisability [2].

2.4. Acrylamide and acrylic acid concentration assays

The procedure used for the assays was modified from [4,5]. Experiments were run with a Chrom 5 gas chromatograph (Laboratorni Pристрои, Praha, Czech Republic) equipped with a flame ionisation detector and 0.3\(\times\)350 cm glass column of 5% Reoplex 400 on Inerton AW-DMCS (Chemopol, Czech Republic) (particle size 0.20-0.25 mm). Flow rates of the helium carrier gas, hydrogen and air were 40, 30, and 500 cm\(^3\) min\(^{-1}\), respectively. An injection volume of 1 \(\mu\)l was used. The oven, injector and detector were maintained at 150, 180, and 190°C, respectively. Acrylamide and acrylic acid were quantified using the absolute calibration method.

2.5. Determination of ammonium ion concentration in culture medium

This was done by the photometric phenol-hypochlorite method [6].
2.6. Amidase assay

Before assay, the cells were sedimented by centrifugation at 5000 $\times$ g for 5 min, resuspended in deionised water and again centrifuged under the same conditions. A pellet resulted which was resuspended in a little deionised water. The cell suspension was subjected to a further centrifugation at 1000 $\times$ g for 1 min to remove cellular aggregates, and the suspension that remained in the supernatant liquid was used for further investigation. The cells thus prepared were incubated with 0.5 g l$^{-1}$ acrylamide at 35°C for 30 min, and acrylamide concentration was determined by gas-liquid chromatographic (GLC) analysis as above. Amidase activity was expressed as milligrams of acrylamide transformed per minute per milligram of dry matter of cells.

3. Results and discussion

Previously, we demonstrated the possibility of EO determination of the enzyme activity of a microbial cell suspension of a *Brevibacterium* sp. and the selectivity of the cellular OS changes toward acrylamide [3]. The most substantial changes were found to occur at the first five frequencies of the orienting field (10 to 1000 kHz). In studying the dependence of the EO effect on the growth period, it therefore appeared reasonable to use $\delta$OD values measured at 502 kHz only.

Our concern here was with the feasibility of determining microbial enzyme activity during batch culture and with comparing these data with those by the conventional method (by the decrease in substrate concentration). Cells of a *Brevibacterium* sp. having amidase activity were chosen for study. The cells were grown for 24 h on BEA with acrylamide (5.0 g l$^{-1}$) as an inducer. Expression of amidase activity was studied during culture of cells on mineral medium with 2.8 g l$^{-1}$ acrylamide. Samples were periodically withdrawn for EO analysis of the cell suspension, for the routine GLC measurement of amidase activity and for the acrylamide, acrylic acid and ammonium ion concentration assays.

We found that 2.8 g l$^{-1}$ acrylamide was hydrolysed in *Brevibacterium* sp. cells to give rise to ammonium ions and acrylamide in a matter of 24 h of growth (Fig. 1A). Maximum acrylic acid content in the medium was seen at 6 h. Fig. 1B shows the dependence of $\delta$OD at 502 kHz on the growth period on mineral medium with acrylamide. It seems likely that acrylic acid is likewise involved in cellular basal metabolism in the presence of acrylamide. That acrylic acid does engage in basal metabolism is evidenced by the ability of the 13 PA cells to grow on mineral medium with acrylic acid as the sole carbon source and by the specific respiration activity of the cells toward acrylic acid, as well as acrylamide [7].

Cellular amidase activity during acrylamide metabolism was determined in the usual way by the change in substrate concentration that occurs during
the microbial hydrolysis of acrylamide to acrylic acid (Fig. 1C). The change in amidase activity correlated with alterations in the cellular EO properties (Fig. 1B). Amidase activity declined after 4 h of growth, possibly due to the progressive accumulation in the medium of acrylic acid ions and ammonium ions which, as shown earlier for a *Rhodococcus rhodochrous* strain [8], have inhibitory activity against amidase.

Since in the cell the metabolism of acrylic acid occurs concomitantly with acrylamide hydrolysis, the alterations in the cellular EO properties may in principle be associated with both the change in amidase activity and the changes in the activities of enzymes that are involved in the further metabolism of acrylic acid. Therefore, it seemed essential to do a special experiment to separate these two effects.

To this end, the cells were grown on mineral medium with 5.2 g l\(^{-1}\) acrylic acid as the sole carbon source. By analogy with the above experiment, samples were withdrawn periodically for EO analysis of the cell suspension and for GLC determination of amidase activity. Additionally, monitoring of concentration changes of acrylic acid in the culture medium was performed (Fig. 2A). The data show that over a period of 48 h, there was a gradual decrease in the medium acrylic acid content.

EO analysis was carried out during cell incubation in deionised water in the presence and absence of acrylamide. The dependence of \(\delta\text{OD}\) on the growth period on mineral medium with acrylic acid is shown in Fig. 2B. No change in \(\delta\text{OD}\) was revealed during cell incubation in acrylamide-containing deionised water.
The change in amidase activity during culture was routinely recorded by the reaction of acrylamide transformation to acrylic acid. The data obtained are summarised in Fig. 2C. The cells exhibited some amidase activity, which remained virtually unchanged within 48 h of growth. Supposedly, the presence of acrylic acid in the culture medium as the sole carbon source inhibited cellular amidase activity. In this case, too, a correlation was apparent between the results of EO analysis of the microbial suspension and those by the conventional approach.

The next step was to investigate the change in amidase activity in cells growing on mineral medium without added carbon sources. As above, samples were withdrawn periodically for EO analysis of the cell suspension and for GLC determination of amidase activity.

EO analysis was carried out during cell incubation in deionised water in the presence and absence of acrylamide. Fig. 3A shows the dependence of δOD on the growth period on mineral medium. The most dramatic δOD changes seen during cell incubation in acrylamide-containing deionised water occurred during the initial hours of growth, then became less marked and virtually disappeared by 48 h. From our viewpoint, this may be due to a change in cellular amidase activity.

Shown in Fig. 3B are the results of GLC determination of amidase activity. Culturing of the cells in mineral medium was accompanied by a reduction in amidase activity. As in the above experiments, a correlation was established between the results obtained for the alterations in the EO properties of the microbial suspension and those obtained for the change in amidase activity.

The data generated from the study of microbial amidase activity can be represented as the difference of the suspension δOD values measured before and after acrylamide incubation. There was qualitative agreement between the results by the EO method and those from the routine determination (Fig. 4).

In sum, we have detected characteristic, amidase activity-dependent alterations in the EO properties of a microbial suspension occurring during cell growth under different conditions. Without doubt, the determination of cellular amidase activity during culture can be done by conventional methods, e.g. by the decrease in substrate concentration or by the amount of product formed. However, it does not always happen that these methods can be carried out with reasonable rapidity, whereas the EO analysis of microbial suspensions has the potential for development of a quick method for determining the enzyme activity of native cells.

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