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Capillary electrophoresis with laser-induced fluorescence detection for studying amino acid uptake by yeast during beer fermentation

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ABSTRACT

The amino acid composition of cultivation broth is known to affect the biomass accumulation, productivity, and vitality of yeast during cultivation. A separation method based on capillary electrophoresis with laser-induced fluorescence (LIF) detection was developed for the determination of amino acid consumption by *Saccharomyces cerevisiae* during beer fermentation. Intraday relative standard deviations were less than 2.1% for migration times and between 2.9% and 9.9% for peak areas. Interday relative standard deviations were less than 2.5% for migration times and between 4.4% and 18.9% for peak areas. The quantification limit was even as low as 62.5 pM which equals to below attomole level detection. The method was applied to study the rate of amino acid utilization during beer fermentation.
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1. Introduction

Nitrogen is one of the main elements in macromolecules of all living organisms. It plays a central role in structure and function, and most organisms have detailed control mechanisms to maintain a constant supply of nitrogen. Yeast is able to use a wide variety of compounds as nitrogen sources, but it prefers ammonia, asparagine, glutamine or glutamate. In the absence of these primary nitrogen sources or if they are present in concentrations low enough to limit growth, other nitrogen sources such as nitrite, nitrate, amides, other amino acids and peptides are used [1].

Amino acid composition of cultivation broth is known to affect the biomass accumulation, productivity, and vitality of yeast during cultivation. In addition, it is known to affect the concentration of flavor-active higher alcohols, vicinal diketones, and esters in beer. The total concentration and composition of amino acids is also important because the use of sugar supplements in cultivation has the effect of reducing the nitrogen/carbon ratio, potentially limiting yeast growth and necessitating the addition of nitrogen supplements [2]. Thus, it is essential for the optimization of process parameters and cultivation performance to monitor amino acid uptake and consumption.

All-malt brewery wort contains a wide variety of natural nitrogen sources, of which amino acids are the most abundant. It contains all the physiologically active amino acids, but there can be differences in individual amino acid concentrations between wort types. However, the overall proportions of amino acids tend to be relatively constant. Amino acids are taken up sequentially, although the exact order of uptake is strain specific [2].

Capillary electrophoresis (CE) is an excellent analysis tool for bioprocess monitoring. It has high resolution, strong separation efficiency, fast analysis times and low consumption of sample and reagents [3]. CE has been used extensively for the analysis of amino acids in different matrices and with different detectors. Amino acids have been analyzed with indirect UV [4], mass spectrometric (MS) [5–8], conductivity [9] and laser-induced fluorescence (LIF) [10–15] detection.

Amino acid separation and identification by direct UV detection is very difficult, because they do not have a chromophore in their structure. It is possible to use indirect UV detection, in which chemicals containing chromophores are added to the background electrolyte (BGE) solution, but the sensitivity is even lower than with direct UV detection [13]. Laser-induced fluorescence (LIF) detection is used widely to improve the sensitivity of CE. It is the most sensitive detection method available with detection limits below 10^{-21} mol, which makes it almost 1000 times more sensitive than UV detection. Most of the analytes do not have native fluorescence and therefore the use of this detection method

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involves attaching fluorescent probe molecules to the analyte. Most fluorescent probes can be designed to contain a reactive moiety capable of coupling to a specific functional group of a biomolecule. With this derivatization procedure it is possible to enhance the selectivity of the detection [16].

Oregon green™ 488 carboxylic acid, succinimidyl ester (OG-SE) is a fluorinated analogue of fluorescein. Conjugates of Oregon green fluorophores are more photostable than those of fluorescein and are less pH sensitive. The excitation and emission maxima of the label are at 496 nm and 524 nm, respectively, which are suitable for use with an argon-ion laser. Reaction of OG-SE with primary and secondary amines creates stable amide and imide linkages, respectively, and the reaction occurs rapidly in mild conditions.

In this study a derivatization technique coupled with capillary electrophoresis separation was developed for the simultaneous monitoring, identification and quantification of amino acids. This method was applied for monitoring the amino acid uptake of yeast during beer fermentation. The results were compared to the consumption of sugars present in the wort and to the production of ethanol during the bioprocess. This study provides a monitoring system with great importance for the optimization of process parameters and cultivation performance.

2. Experimental

2.1. Chemicals

Stock solutions of amino acid standards were prepared in 18 mΩ deionized water (Millipore, Bedford, MA, USA) at 10 mM concentration. Oregon green® 488 carboxylic acid, succinimidyl ester 6-isomer (Molecular Probes Inc., Eugene, OR, USA) was prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany) as a 2 mM solution.

L-isoleucine (≥99.5%), L-serine (≥99%), L-leucine (≥99%), L-histidine (≥99.5%), L-threonine (≥99%), L-tryptophan (≥99%), L-lysine (≥98%), L-methionine (≥99%), L-arginine (≥99%), L-glutamic acid (≥99%), L-cysteine (≥99.5%), L-asparagine (≥99%), L-aspartic acid (≥99%), L-proline (≥99%), and L-glutamine (≥99%) were purchased from Fluka (Buchs, Switzerland). L-tyrosine (≥99%), L-phenylalanine (≥99%), L-alanine (≥99%) and glycine (≥99.7%) were from Merck (Darmstadt, Germany). DL-valine (≥97%) was from Sigma-Aldrich.

Background electrolyte (BGE) solutions were prepared from sodium tetraborate decahydrate (≥99.5%), sodium dodecyl sulfate (SDS) (≥99%), 18-crown-6-ether (99%), β-cyclodextrin (≥97%), and CHAPSO (≥98%), which were purchased from Sigma-Aldrich. A 0.1 M sodium hydroxide solution for the capillary conditioning was from FF-Chemicals Ab (Haukipudas, Finland).

2.2. Instrumentation

All the measurements were made with a P/ACE MDQ capillary electrophoresis system equipped with an LIF detector (Beckmann Coulter Inc., Fullerton, CA, USA). The LIF detector had an argon-ion laser with 488 nm excitation and 520 nm emission wavelengths. Bare fused-silica capillaries (Teknolab, Trollasen, Norway) with inner diameter 50 μm, outer diameter 360 μm, and total length 60 cm (50 cm to the detector) were used. The capillaries were conditioned before use with 0.1 M NaOH, milli-Q purified water, and BGE for 10 min each. Voltage (+30 kV) was then applied for 5 min. Between analyses, capillaries were rinsed with 0.1 M NaOH, 18 mΩ deionized water and with BGE for 2, 1 and 2 min, respectively. Data was collected and processed with 32Karat software (Beckmann Coulter Inc., Fullerton, CA, USA).

2.3. Analysis conditions

BGE was prepared with 50 mM sodium tetraborate, 30 mM SDS and 20 mM 18-crown-6-ether in milli-Q purified water. The pH of the solution was 9.0. Before analysis, the BGE was degassed by ultrasound and filtered through a 0.45 μm Acrodisc GHP syringe filter (Pall Life Science, Ann Arbor, MI, USA).

Standards and samples were injected by using 0.5 psi pressure for 10 s, giving rise to an injection volume of 8 nL. Separation was carried out at +30 kV at constant capillary temperature +15 °C. Sample cartridge temperature was set at +15 °C. The analytes were detected with the LIF detector using an excitation wavelength of 488 nm and emission wavelength of 520 nm. Concentrations of sugars and alcohols were analyzed by a Waters Alliance 2690 HPLC system (Milford, MA, USA) with Waters 2487 dual wavelength UV (210 nm) detector and Waters 2414 differential refractometer with an injection volume of 10 μL. The columns were an ICSep ION-310 Fast Analysis Column (150 mm × 6.5 mm, Transgenomic, Inc., Omaha, NE, USA) connected to an ICSep ICE-ORH-801 Column (300 mm × 6.5 mm, Transgenomic). The eluent was 5 mM H_2SO_4 (Titrisol, Merck), flow rate 0.5 mL/min and analysis temperature +55 °C. The optical density of the sample was measured by a Shimadzu UV-1201 spectrometer (Kyoto, Japan) at wavelength 600 nm (=OD_{600}). OD_{600} is a measure of cell density. The cell dry weight (CDW) was determined by filtering 20 mL of sample through a measured filter paper, washing it with distilled water and drying it in an oven (105 °C) for at least 16 h. After that the cells were weighed with the filtration paper and the CDW was calculated.

2.4. Cultivation conditions and sampling

To obtain a pre-culture, *Saccharomyces cerevisiae* strain VTT A-6035 (VTT, Finland) was propagated as a 2 L culture in a Biostat

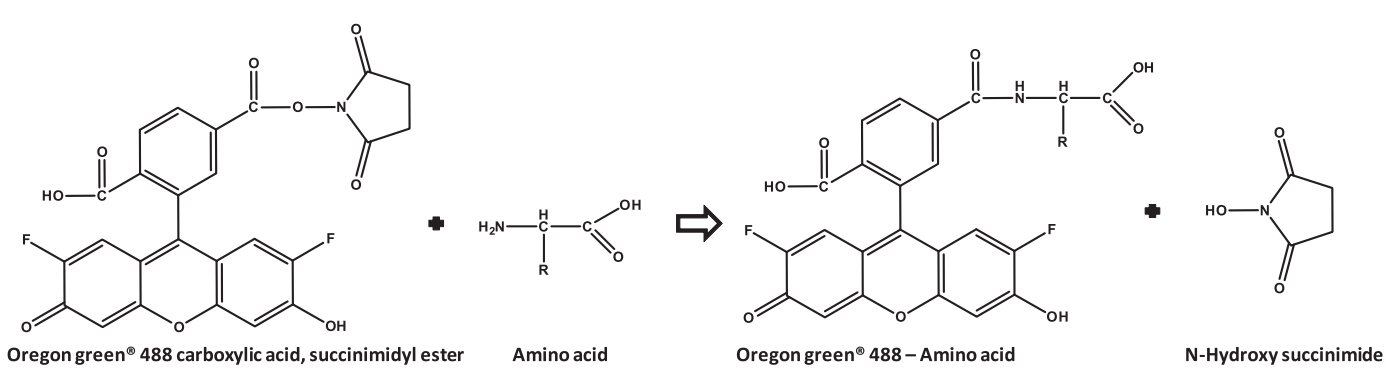

Fig. 1. Labeling reaction of Oregon green 488® succinimidyl ester (6-isomer) with an amino acid.

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