Electronic Journal of Biotechnology 18 (2015) 252–255

HOSTED BY

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

A rapid method for an offline glycerol determination during microbial fermentation

Jennifer Kuhn [a], Hagen Müller [b], Denise Salzig [a,*], Peter Czermak [a,b,c,d]

[a] Institute of Bioprocess Engineering and Pharmaceutical Technology, University of Applied Sciences Mittelhessen, Wiesenstrasse 14, 35390 Giessen, Germany
[b] Departments of Chemical Engineering, Kansas State University, Manhattan, KS, USA
[c] Faculties of Biology and Chemistry, University of Giessen, Germany
[d] Fraunhofer Institute for Molecular Biology and Applied Ecology, Project Group "Bioresources", Winchesterstrasse 3, 35394 Giessen, Germany

ARTICLE INFO

Article history:
Received 27 February 2014
Accepted 30 October 2015
Available online 7 February 2015

Keywords:
Glycerol determination
Fermentation broth
Hantzsch reaction
Malaprade reaction

ABSTRACT

Background: The purpose of this work was to find a rapid method for glycerol detection during microbial fermentations. The method requirements were, first, to avoid sample pretreatment, and second, to measure glycerol precisely especially out of fermentation broth.
Results: This was achieved by combining two reaction principles — the Malaprade reaction and the Hantzsch reaction. In the Malaprade reaction, glycerol is converted into formaldehyde. This forms a dye in the Hantzsch reaction after which adsorption is than detected. The subsequent assay was investigated with two different fermentation media, a chemically undefined and a chemically defined media, used for *Pichia pastoris* fermentation. In both media, as well as in real fermentation samples, glycerol content could be reproducibly detected with the method. Moreover, measurements were more precise than using a standard glycerol detection kit.
Conclusions: With this rapid assay, glycerol could be detected easily in microbial fermentation broth. It is reliable over a wide concentration range including advantages such as an easy assay set-up, a short assay time and no sample pretreatment.

1. Introduction

For a multitude of bacterial or yeast fermentations, glycerol is a frequently used carbon source. Most standard methods used to determine the glycerol content in fermentation media use the high performance liquid chromatography (HPLC) with a refractive index detector. Due to the number of samples taken during the fermentation and the need of a complex sample preparation, the HPLC measurement is not only time-consuming but also cost-intensive. In addition, several glycerol detection kits are available which are based on enzymatic reactions and spectrometric detection of a dye. Unfortunately these assays are often developed for *e.g.* food samples, plasma or serum and are not suitable for fermentation samples. Thus the focus in this work was to find a simple and fast glycerol detection method without the need for pretreatment of the fermentation samples. This was achieved by combining the Malaprade reaction and the Hantzsch reaction. This choice was done because methods based on the Malaprade reaction are the ones that are most widely used for the detection of 1,2-diols or related compounds including glycerol. In this reaction, glycerol is converted with periodate into formaldehyde (Fig. 1) [1].

Afterwards, the Hantzsch reaction is used for the detection of formaldehyde. In this reaction, acetylacetone and ammonia are used to form a dye in connection with formaldehyde (Fig. 2). This dye is 3,5-diacetyl-1,4-dihydrolutidine (DDL), which can be measured at 410 nm in a spectrophotometer [2].

The assay can be done in a standard spectrophotometer or a microplate reader. Using the microplate reader has the advantage that more samples (*e.g.* 96 or 384, dependent on the well plate) can be measured at once.

2. Materials and methods

2.1. Fermentation media

The chemically undefined medium BMGY contained the following ingredients: 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 11.9 g L^{-1} KH_2PO_4 and 2.1 g L^{-1} K_2HPO_4 (pH 6.0) dissolved in water. pH adjustment was done with 1 M NaOH if needed. After autoclaving (121°C, 20 min), sterile-filtrated yeast nitrogen base (final concentration 13 g L^{-1}) and sterile-filtrated biotin (final concentration 4 g L^{-1}) were added to the solution [3].

* Corresponding author.
E-mail address: denise.salzig@kmub.thm.de (D. Salzig).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.01.005

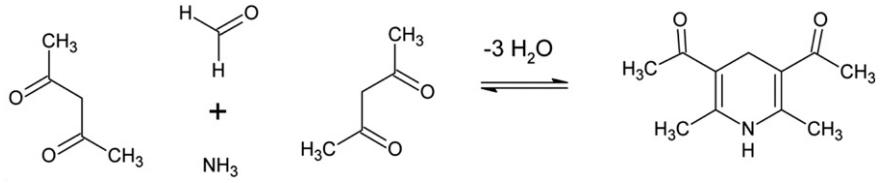

Fig. 1. Reaction scheme of the Malaprade reaction. In this reaction, sodium periodate is used to convert glycerol into formaldehyde.

The chemically defined medium FM22 consisted of 42.9 g L^{-1} KH_2PO_4, 5.0 g L^{-1} $(NH_4)_2SO_4$, 1.0 g L^{-1} $CaSO_4 \cdot 2H_2O$, 14.3 g L^{-1} K_2SO_4, 5.2 g L^{-1} $Na_3C_6H_5O_7$ and 11.7 g L^{-1} $MgSO_4 \cdot 7H_2O$ (pH 5.0). pH adjustment was done with ammonia solution (30%, Carl Roth) [4]. The medium was autoclaved at 121°C for 20 min. Additionally 8.7 mL L^{-1} of the sterile-filtrated trace-metal solution was added. The trace-metal solution (PTM1) contained 6.0 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.08 g L^{-1} NaI, 3.0 g L^{-1} $MnSO_4 \cdot H_2O$, 0.2 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.02 g L^{-1} H_3BO_3, 0.5 g L^{-1} $CoCl_2$, 20 g L^{-1} $ZnCl_2$, 65 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.2 g L^{-1} biotin and 5.0 mL L^{-1} H_2SO_4 [4].

Autoclaved glycerol was added to both media in different concentrations for assay validation. During the fermentation, the initial glycerol content in the medium was 15 g L^{-1}.

2.2. Fermentation of Pichia pastoris

For the fermentation, the Pichia Pink Expression System from Invitrogen was used [3]. A 50 mL overnight-culture was done in shaking flasks. 7 μL antifoam (Struktol J673A, Schill + Seilacher) was added to the medium. Inoculation of the pre-culture was done with 1 mL of a cryo-culture (OD_{600} ~ 5). The pre-culture was grown at 30°C and 250 rpm for approx. 16 h. The fermentation was performed in a 20 L stirred tank reactor (20 L Bench Top Reactor, Applikon, Schiedam, Netherlands). Working volume was 7 L. The fermentation medium was prepared as described above. Before inoculation 1 mL sterile antifoam was added. The fermentation parameters were set to 30°C, 800 rpm and an aeration rate of 1 vvm. The medium was inoculated with the pre-culture to a final OD_{600} of 0.1. Cells were grown for 24 h.

2.3. Sampling

For the detection of glycerol and biomass, samples were taken from the reactor. Therefore every h 1 mL fermentation broth was taken out of the vessel. 500 μL was immediately used for biomass determination. The other 500 μL was centrifuged (5 min, 5000 × g). The supernatant was transferred into a new tube and stored at -20°C for glycerol determination.

2.4. Biomass determination

The growth of the culture was controlled by measuring the optical density at 600 nm (OD_{600}) of the fermentation broth with a standard spectrophotometer. Samples were diluted with fermentation medium until OD_{600} reaches values between 0.1 and 1. With a correlation between the optical density and the biomass dry weight, the biomass content was calculated (CDW in g L^{-1} = 0.47·OD_{600}). Therefore a concentration series of *P. pastoris* biomass was prepared. Each sample (15 mL) of the concentration series was both measured at OD_{600} and for CDW (cell dry weight). For CDW determination 10 mL of the sample was centrifuged, washed with ddH_2O, centrifuged again and resuspended in ddH_2O. The solution was transferred to a weighted beaker and dried at 100°C until constant weight.

2.5. Glycerol detection combining the Malaprade reaction and the Hantzsch reaction

For the assay, two reagents were needed. Reagent I, the periodate reagent, consisted of 18 mg mL^{-1} sodium periodate (Merck) dissolved in distilled water with 10% (v/v) acetic acid (Merck). For preparation purposes, sodium periodate was first dissolved in water. After the addition of acetic acid and adequate mixing, 77 mg mL^{-1} ammonium acetate (VWR) was added. The amount of sodium periodate in this reagent was calculated for a calibration curve from 50 mg L^{-1}–200 mg L^{-1} glycerol. Reagent II, the acetylacetone reagent, was composed of 1% (v/v) acetylacetone (VWR) in isopropyl alcohol (Roth). This reagent had to be stored in the dark.

The measurement was executed in a microplate reader (Synergy HT BioTek, Winooski, USA). Therefore, an amount of 40 μL sample (cell-free supernatant of the fermentation) was pipetted into each well of a standard 96 well plate (Greiner). Then, 40 μL Reagent I was added and mixed adequately. After an incubation time of 10 min, 125 μL Reagent II was pipetted into each well and mixed adequately. The absorption at 410 nm was measured over a period of 25 min in the plate reader. The glycerol content was calculated with Eq. (1), based on a glycerol standard curve (50 mg L^{-1}–200 mg L^{-1}).

$$\text{glycerol content}\left[\frac{\text{mg}}{\text{l}}\right] = \frac{\Delta A_{\text{sample(25 min-15 min)}} - \Delta A_{\text{blank(25 min-15 min)}}}{\text{slope of the calibration curve}\left[\frac{\text{l}}{\text{mg}}\right]} \quad \text{[Equation 1]}$$

The assay can also be performed in a cuvette using a spectrophotometer. In this instance, 5-fold the amount of sample and reagents must be used.

2.6. Measurement of glycerol using a standard assay

The assay was performed in a microplate reader according to the microplate assay procedure in the Glycerol GK Kit manual from Megazyme [5]. In this assay, the glycerol is phosphorylated with the use of adenosine-5′-triphosphate (ATP). The formed adenosine-5′-diphosphate (ADP) is used for the phosphorylation of D-glucose which is oxidized with the formation of nicotinamide-adenine

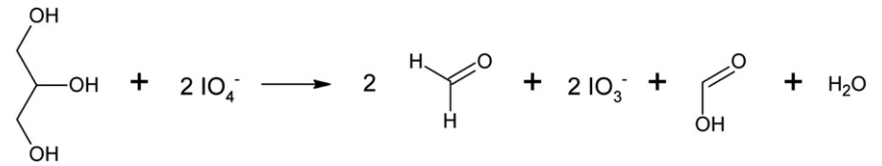

Fig. 2. Reaction scheme of the Hantzsch reaction. In this reaction, formaldehyde is converted into DDL with ammonia and acetylacetone. This dye can be measured at 410 nm.