Journal of Microbiological Methods 82 (2010) 288–295

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

UPLC/MS based method for quantitative determination of fatty acid composition in Gram-negative and Gram-positive bacteria

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ARTICLE INFO

Article history:
Received 3 June 2010
Received in revised form 2 July 2010
Accepted 2 July 2010
Available online 16 July 2010

Keywords:
Fatty acid quantification
Ultra performance liquid chromatography
Electrospray ionization mass spectrometry
Escherichia coli
Lactococcus lactis

ABSTRACT

Quantitative fatty acid composition of microorganisms at various growth space points is required for understanding membrane associated processes of cells, but the majority of the relevant publications still restrict to the relative compositions. In the current study, a simple and reliable method for quantitative measurement of fatty acid content in bacterial biomass without prior derivatization using ultra performance liquid chromatography–electrospray ionization mass spectrometry was developed. The method was applied for investigating the influence of specific growth rate and pH on the fatty acid profiles of two biotechnologically important microorganisms — Gram-negative bacteria *Escherichia coli* and Gram-positive bacteria *Lactococcus lactis* grown in controlled physiological states. It was found that the membranes of slowly growing cells are more rigid and that the fatty acid fraction of the cells of *L. lactis* diminishes considerably with increasing growth rate.

1. Introduction

Monomer composition of cells and its changes are important parameters which make possible identification and monitoring of physiological states of microorganisms in different growth conditions. Detailed identification and characterization of the physiological states is achieved by the construction of metabolic models which are able to explain and predict the behavior of a microorganism. For functioning of these models, quantitative data about cell composition should be determined for all the studied physiological states.

This has been rarely achieved because of deficiency and complexity of quantitative analytical methods, and a general assumption that variations in biomass composition are relatively small (Pramanik and Keasling, 1997). Indeed, it has been shown that several macromolecular fractions maintain sufficiently constant monomer composition, but there are also notable exceptions to this constancy like the content of fatty acids of the membrane (Pramanik and Keasling, 1998). It has been demonstrated that fatty acid composition of bacterial membranes can be largely varied depending on environmental conditions (Guerzoni et al., 2001; Guillot et al., 2000; Kimoto-Nira et al., 2009; Marr and Ingraham, 1962; Shaw and Ingraham, 1965; Van de Guchte et al., 2002). However, the data are rarely quantitative and, except for *Escherichia coli* (Arneborg et al., 1993; Pramanik and Keasling, 1998; Shokri et al., 2002), the knowledge concerning the effect of specific growth rate is essentially missing.

Fatty acids of bacteria are primarily analyzed as methyl esters (ME) employing gas chromatography (GC) (Basconcillo and McCarry, 2008; Dionisi et al., 1999; Eder, 1995; Rozes et al., 1993). However, complete separation of many important *cis*- and *trans*-isomers of fatty acids is not possible using GC (Ratnayake and Beare-Rogers, 1990). In addition, methylation of fatty acids may be quite troublesome. Incomplete conversion of fatty acids to their corresponding ME and possible side reactions (mainly hydrolysis) during methylation process might lead to inadequate quantitative results. Due to low stabilities of some fatty acids (hydroxy- and cyclopropane fatty acids), the results obtained might vary more than ten times dependent on the methylation method used (Basconcillo and McCarry, 2008).

Hence, it is obvious that elimination of the derivatization step should be considered highly advantageous, as it makes the methods more accurate, rapid, cost-effective and easier to use. This is especially important for the methods used routinely or with high throughput (Dillon et al., 2008). Analysis of underivatized fatty acids can be accomplished by the application of liquid chromatography (LC) combined with mass spectrometer (MS), due to its high sensitivity and selectivity (Carrier and Parent, 2001). As a potential tool for fatty

Abbreviations: APCI, Atmospheric pressure chemical ionization; APE, Absolute percent error; CFA, Cyclopropane fatty acids; EI, Electron ionization; ESI-MS, Electrospray ionization mass spectrometry; FAME, Fatty acid methyl esters; FFA, Free fatty acids; HPLC, High performance liquid chromatography; LAB, Lactic acid bacteria; LOD, Limit of detection; LOQ, Limit of quantification; PBS, Phosphate-buffered saline; RSD, Relative standard deviation; SFA, Saturated fatty acids; TIC, Total ion chromatogram; TOF, Time of flight; UFA, Unsaturated fatty acids; UPLC, Ultra performance liquid chromatography.

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doi:10.1016/j.mimet.2010.07.006

acid analysis, LC/MS gained its advantages with the development of soft ionization techniques, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) (Bogusz, 1999; Kerwin et al., 1994; Niessen, 1998). Sensitivity of the method can be further enhanced with the use of ultra performance liquid chromatography (UPLC) in place of high performance liquid chromatography (HPLC) (Chu et al., 2009). The reason of increased sensitivity lies in the diameter of stationary phase particles, which is decreased to less than 2 μm in case of UPLC. As efficiency of column is inversely related to particle size, UPLC has better resolution and sensitivity than HPLC.

During the last decade, HPLC/MS and UPLC/MS based methods for free fatty acid (FFA) analysis have proven to be simple, rapid (Carrier and Parent, 2001; Chu et al., 2009; Perret et al., 2004; Zehethofer et al., 2008), accurate (Chu et al., 2009), sensitive (Chu et al., 2009; Perret et al., 2004), reproducible (Carrier and Parent, 2001; Rigol et al., 2003) and able to separate several important *cis*- and *trans*-isomers of fatty acids (Zehethofer et al., 2008) in case of various other than biomass substances.

The lack of information concerning bacteria could be therefore considered even somewhat unexpected. Only a limited number of papers have been published on fatty acid analysis in bacteria with LC/MS (Nichols and Davies, 2002) and to our knowledge, analysis of bacterial fatty acids without prior derivatization with LC/MS has not yet been reported. The aim of the current study was to develop a method enabling quantitative determination of bacterial fatty acid composition without prior derivatization.

The developed method was applied for assaying the fatty acid compositions of Gram-positive bacteria *Lactococcus lactis* and Gram-negative bacteria *E. coli* in fully controlled environmental conditions using changestat (Adamberg et al., 2009) cultivation methods. The correlation between fatty acid composition and specific growth rate was examined in both bacteria and in *L. lactis* the effect of environmental pH was also studied.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. lactis IL1403 culture was kindly provided by Dr. Ogier from INRA, France.

D-stat cultivations (see details in Lahtvee et al., 2009) at dilution rate 0.2 h^{-1} and A-stat cultivations (see details in Adamberg et al., 2009) with initial dilution rate 0.1 h^{-1} and acceleration rate 0.01 h^{-2} were carried out. Fermentations were performed under anaerobic conditions (N_2 environment) at a temperature of 34 °C and with an agitation speed of 300 rpm. The pH of the culture was kept at 6.4 ± 0.1 using 2 M NaOH.

The cultivation medium was composed of 70% HAM-medium (Invitrogen, USA) and 30% medium as published in Adamberg et al. (2009) (final glucose concentration was 3.5 g L^{-1}). The medium composition was as follows (g L^{-1}): glucose, 3.5114; alanine, 0.0782; arginine, 0.1852; asparagine, 0.0735; aspartate, 0.0723; cysteine, 0.0636; glutamate, 0.0703; glutamine, 0.1322; glycine, 0.0578; histidine, 0.0597; isoleucine, 0.1018; leucine, 0.2072; lysine, 0.1576; methionine, 0.0407; phenylalanine, 0.0860; proline, 0.0917; serine, 0.1634; threonine, 0.0758; tryptophane, 0.0164; tyrosine, 0.0295; valine, 0.1072; K_2HPO_4, 0.9; KH_2PO_4, 0.75; $NaHCO_3$, 0.9878; NaCl, 5.5803; $CaCl_2$, 0.0278; $MnSO_4$ H_2O, 0.0014; $ZnSO_4$ $7H_2O$, 0.0011; $CoSO_4$ $7H_2O$, 0.0003; $CuSO_4$ $5H_2O$, 0.0003; $(NH_4)_6$ Mo_7O_{24} $4H_2O$, 0.0003; $FeSO_4$ $7H_2O$, 0.0007; KCl, 0.1565; $MgCl_2$, 0.0581; Na_2HPO_4, 0.0994; pyridoxine, 0.0006; biotin, 0.0003; choline, 0.0098; vitamin B12, 0.001; inositol, 0.0126; folic acid, 0.0012; niacin, 0.0003; pantothenic acid, 0.0007; riboflavin, 0.0003; thiamine, 0.0005; hypoxanthine, 0.0033; linoleic acid, 0.0001; lipoic acid, 0.0001; phenol red, 0.0008; putrescine, 0.0001; sodium pyruvate, 0.077; and thymidine, 0.0005.

E. coli K12 MG1655 (λ^- F^- *rph-1Fnr*$^+$) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany.

A-stat cultivations with initial dilution rate 0.1 h^{-1} and acceleration rate 0.01 h^{-2} were carried out. Fermentations were performed under aerobic conditions (O_2 environment) at a temperature of 37 °C and with an agitation speed of 600–800 rpm. The pH of the culture was kept at 7.0 ± 0.1 using 2 M NaOH.

The cultivation medium composition was used as follows (g L^{-1}): glucose, 5; $MgSO_4$ $7H_2O$, 0.5; NH_4Cl, 3.5; K_2HPO_4, 2; $FeSO_4$ $7H_2O$, 0.005; $MnSO_4$ H_2O, 0.0005; $CaCl_2$ $2H_2O$, 0.005; $ZnSO_4$ $7H_2O$, 0.002; $CoSO_4$ $7H_2O$, 0.0006; $CuSO_4$ $5H_2O$, 0.0005; $(NH_4)_6$ Mo_7O_{24} $4H_2O$, 0.0026.

2.2. Chemicals

Hexadecanoic acid (palmitic acid, C16:0), *cis,cis*-9,12-octadecadienoic acid (linoleic acid, C18:2), *trans*-9-octadecenoic acid (elaidic acid, C18:1,*trans*), *cis*-9-octadecenoic acid (oleic acid, C18:1,*cis*) and *cis*-11,12-methyleneoctadecanoic acid (phytomonic acid, C19:0,) were purchased from Cayman Chemical (Ann Arbor, Michigan, USA), *cis*-9-hexadecenoic acid (palmitoleic acid, C16:1) and tetradecanoic acid (myristic acid, C14:0) from Sigma (St. Louis, MO, USA). The purity of all the fatty acids was ≥98%. Stock standard solutions containing 0.125–0.5 mg mL^{-1} of various free fatty acids (FFA) were prepared in ethanol and stored at −80 °C. Necessary dilutions were made with ethanol prior to analysis. Identification and quantification of fatty acid methyl esters (FAME) with GC/MS were based on Supelco F.A.M.E. C4–C24 mixture (St. Louis, MO, USA). Potassium hydroxide (pellets) and formic acid were obtained from Fluka (Buchs, Switzerland), methanol, 0.5 M sodium methoxide in methanol, glyceryl tripalmitate and hexane from Sigma (St. Louis, MO, USA), acetonitrile from Rathburn Chemicals (Walkerburn, Scotland) and chloroform from Scharlau Chemie S.A. (Sentmenat, Barcelona, Spain). Ethanol (96%) was purchased from Rakvere Distillery (Estonia). Distilled water was further purified by passage through a Millipore Simplicity system (Millipore, Molsheim, France).

2.3. Sample preparation for analysis with UPLC/ESI-MS

Cells for fatty acid analysis with UPLC/ESI-MS were harvested by centrifugation (15,500 g, 5 min) and washed once with PBS buffer. The precipitated biomass was stored at −20 °C until analysis and resuspended in 0.1 mL of PBS buffer directly before analysis. Approximately 1.0 mg dry mass of bacteria was needed for the analysis. Bacterial biomass was subjected to basic hydrolysis, which was carried out in 15 mL sealed glass tubes with 500 μL of a 5% KOH (w/v) in 80% MeOH (v/v) solution for 3 h at 80 °C. After saponification, the pH of the solution was acidified with 1/8 of the volume of formic acid and the free fatty acids were extracted with 500 μL of hexane/chloroform (4:1; v/v). After vortexing, an aliquot of the upper hexane/chloroform layer was transferred to a glass vial. Solvent was evaporated under a gentle stream of nitrogen and the dried extracts were dissolved in ethanol. The samples were analyzed immediately or stored at −20 °C.

2.4. Sample preparation for analysis with GC/MS

Cells for fatty acid analysis with GC/MS were harvested by centrifugation (17,500 g, 15 min) and washed once with dH_2O. The precipitated biomass was stored at −20 °C until analysis and resuspended in 0.1 mL of dH_2O directly before analysis. Approximately 5.0 mg dry mass of *E. coli* and 10.0 mg dry mass of *L. lactis*