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Quantification of solid cell material by detection of membrane-associated proteins and peptidoglycan

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Abstract

Quantification of solid cell material (cell debris) is necessary for the optimisation of the efficiency of bioseparations. Cell debris can be quantified by detection of a component present in the cell wall that can act as a marker for cell debris. Membrane-associated proteins have previously been used as a marker for cell debris. This marker was quantified by SDS–PAGE with densiometry. In this paper cell debris quantification methods are presented that are faster and more accurate, i.e. membrane-associated protein quantification with the Protein 50 Labchip® of Agilent Technologies, or that make use of peptidoglycan as marker for cell debris, i.e. a spectrophotometric muramic acid assay.

Keywords: Cell debris; *Escherichia coli*; *Pseudomonas putida*; Acetaldehyde; Muramic acid; Peptidoglycan

1. Introduction

Microorganisms can produce proteins and other products that are of economical interest. These products are either excreted into the medium or accumulated inside the cells. The product may become solid, e.g. crystal, precipitate or inclusion body, when it is produced above its saturation concentration or accumulated in specific compartments inside the cell. Some examples of solid products produced by microorganisms are PHA-granules produced by *Pseudomonas putida* [1], IGF produced by *Escherichia coli* [2], β-carotene crystals produced by *Blakeslea trispora* [3] and α-glucosidase inclusion bodies produced by *E. coli* [4]. Selective recovery and purification of these solid microbial products is an important challenge for large-scale production. In general, recovery of non-excreted products involves cell disruption followed by separation of the product from other cell material. Chemical cell disruption methods use enzymatic treatment, surfactants, solvents and/or other chemicals to degrade the cell wall. Mechanical disruption methods, such as high-pressure homogenisation, bead mill and French press, make use of high shear forces to rupture the cell wall. Cell disruption yields a mixture of product either in dissolved or particulate form, dissolved cell components and solid cell components (cell debris). There are a number of methods available for the purification of solid products from cell debris and dissolved cell components, i.e. extraction [5], filtration [6], centrifugation [7], interfacial partitioning [8] and selective flotation [2]. The efficiency of separation in these methods is measured by quantification of the fractions of cell debris, dissolved cell components and product in the product stream relative to the waste and/or feed stream. Especially when the product is a solid, it is important to trace cell debris throughout the production process; thus methods for cell debris quantification are required for measurement of the process efficiency.

A large part of the solid cell material in microorganisms can be ascribed to the cell wall (Fig. 1), which consists of peptidoglycan (Fig. 2), lipids, phospholipids, proteins and lipoproteins. Peptidoglycan is a polymer composed of muramic acid, glucosamine and a short peptide. The composition of this short peptide is organism-dependent and generally consists of three to five amino acids of which D-alanine is specific for peptidoglycan [9]. One of the components present in cell debris can be selected to act as its marker. This marker will often be sufficient for quantification of the cell debris fraction in various process streams. There are numerous methods described in literature for the quantification of biomass components in whole cell cultures. These methods are, e.g. muramic acid,

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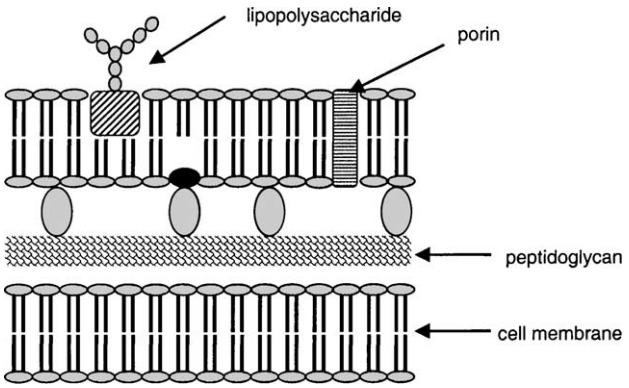

Fig. 1. The cell wall of a Gram-negative microorganism.

diaminopimelic acid and D-alanine quantification with gas chromatography (GC) [10], muramic acid quantification with gas–liquid chromatography [11], fluorescence derivation of muramic acid followed by HPLC quantification of the derivative [12,13], HPLC quantification of phenylthiocarbamyl muramic acid and glucosamine [14], enzymatic quantification of D-alanine [9], muramic acid quantification by GC in combination with ion trap tandem mass spectrometry [15,16], immunochemical detection of polysaccharides with antibodies [17], peptidoglycan quantification with silkworm larvae plasma [18], spectrophotometric glucosamine quantification [19], enzymatic quantification of D-lactic acid [20] and dry weight (DW) measurement. Most methods are time-consuming, laborious and often need complex pre-treatment before the actual analysis. Dry weight measurements, on the other hand, are fast but give an inaccurate measure due to the presence of material other than cell debris that is included in the measurement. The techniques listed above have been generally used to detect microbial contamination within samples without the construction of cell debris mass balances throughout bioprocesses. Furthermore, the simple application of these techniques does not permit an estimation of the size of the contaminating cell debris fragments at any point in the process.

Wong et al. [21] developed a simple method using SDS–PAGE analysis of membrane-associated proteins in combination with densiometry for the quantification of cell debris in samples of mixed particulates. They combined their quantification method with cumulative sedimentation analysis (CSA), which is a technique that makes use of the relation between particle size and particle settling velocity in a centrifugal field to determine the cell debris particle size. The work demonstrated that “markers” for mixed particle populations could be used in combination with CSA, instead of DW measurements, to measure individual size distributions in bioprocessing applications. However, SDS–PAGE in combination with densiometry is a time-consuming analysis technique, so we propose to automate the method of Wong et al. [21] by conducting SDS–PAGE on the Protein 50 Labchip® of Agilent Technologies for the detection of the membrane-associated proteins. This fully automated small-scale analysis method is expected to be much less dependent on the skill of the operator than conventional SDS–PAGE with densiometry.

An alternative for the quantification of membrane-associated proteins as a marker for cell debris is the quantification of peptidoglycan or one of its components [18]. When peptidoglycan is hydrolysed, muramic acid, glucosamine and a short peptide containing D-alanine are dissolved. Muramic acid can be quantified with the simple method of Barker and Summerson [22]. In their method muramic acid is cleaved into lactic acid and 2-amino-D-glucose by acid treatment. Subsequently, lactic acid is hydrolysed into acetaldehyde, carbon monoxide and water [23] followed by a simple chemical reaction between acetaldehyde, copper and *p*-phenylphenol to form chromagen, which can be quantified with a spectrophotometer. This method is more accurate than the enzymatic assay for lactic acid due to the lack of specificity in lactic dehydrogenase that is used in the enzymatic assay [24]. Taylor [25] improved the method of Barker and Summerson and the improved assay by Hadzija [26] to a method that is at least two times as sensitive (less than 3.3% deviation), which can accurately quantify muramic acid concentrations from 0 to 150 nM. As a possible alternative to the method of Barker and Summerson, acetaldehyde can be quantified by GC.

In this work, we compare three methods for the quantification of cell debris: (1) SDS–PAGE on a Protein 50 Labchip® of Agilent Technologies, (2) GC detection of acetaldehyde and (3) the colorimetric muramic acid assay of Barker and Summerson. Optical density measurements were used as reference for all methods. The analysis methods were used in combination with CSA in order to test the analysis

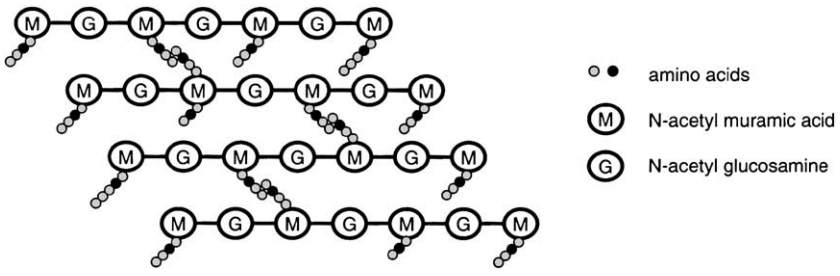

Fig. 2. Structure of peptidoglycan in *E. coli* consisting of *N*-acetyl glucosamine (G) and *N*-acetyl muramic acid (M) and short peptides.

methods on samples containing different particle concentrations and particle size distributions. Whole cells of *E. coli* and *P. putida* were used instead of cell debris because whole cells are more defined than cell debris and optical density measurements can be used as a reference method in the size range of whole cells, which is not the case with cell debris.

2. Methodology

2.1. CSA

Wong et al. [21] used cumulative sedimentation analysis to quantify the size distribution of cell debris. The method is based on the theory that large particles obtain a higher settling velocity under gravity (v_g) than small particles for laminar conditions, which can be explained by Stokes' law (Eq. (1)).

$$v_g = \frac{H}{t_e} = \frac{D^2 \Delta\rho g}{18\eta} \quad (1)$$

Here, H is the settling height; t_e, effective settling time; D, Stokes diameter; $\Delta\rho$, density difference between the particle and the fluid; g, gravitational acceleration and η, viscosity. The effective settling time is defined as the time needed for a particle with diameter D to travel distance H under gravity. All parameters in Eq. (1) are known except for the term $D^2\Delta\rho$, hence a relation between particle settling velocity (or effective settling time) and the Stokes diameter can be obtained when the density difference between the particles and fluid is known. During sedimentation of a homogeneous mixture of particles, the particle mass fraction that settled to the concentrate (F) will increase with increasing effective settling time (t_e). The settled particle mass fraction (F) consists of particles that have a settling velocity larger than H/t_e and particles with a settling velocity smaller than H/t_e. The last class of particles is present in the sediment because the particles were at some intermediate height in the column before sedimentation started. The particles in the first class are referred to as the oversized settled particles. It is thus possible to obtain a cumulative curve for the oversized settled particle mass fraction (W) by discriminating between the two classes using Allen's relation [27] that is shown as Eq. (2):

$$W(t_e) = F - t_e \frac{dF}{dt_e} = F - \frac{dF}{d\ln t_e} \quad (2)$$

The method for cell debris quantification that is coupled to CSA thus must be capable of measuring the cell debris mass fraction that has settled from the settling zone to the concentrate. Here, we discuss the use of optical density measurement, peptidoglycan quantification with GC, a spectrophotometric assay for muramic acid quantification and membrane-associated protein quantification with SDS–PAGE on the Protein 50 Labchip® of Agilent Technologies. In the following three sections the methodology of these detection methods is discussed.

2.2. Optical density

Optical density measurement is based on the absorbance of light by particles in a fluid, which is proportional to the particle concentration according to the Lambert–Beer law (Eq. (3)):

$$A_\lambda = \varepsilon c l \quad (3)$$

where A_λ is the light absorbance at wavelength λ; ε, extinction coefficient; c, particle concentration and l, path length of the light through the solution. Ideally, light absorption is proportional to the projected cross-sectional area of the particles. However, when the particle size approaches the wavelength of light this ideal behaviour ceases to hold [28]. This change in the relation between absorbance and particle size can be accounted for by determination of the extinction coefficient. For polystyrene particles, used to mimic the absorption behaviour of inclusion bodies by Taylor et al. [29], the relation between the extinction coefficient and particle size is linear for particle diameters above 500 nm, which shows that the relation between particle concentration and absorbance is linear as well. For polystyrene particles with a diameter below 500 nm the relation is strongly non-linear. In this latter regime the relation between the extinction coefficient and particle diameter should be known in order to relate absorbance to particle concentration. In fermentation fluids, the majority of cells is often much larger than 500 nm. When cells are disrupted however, the particle size becomes smaller than 500 nm; thus tedious extinction coefficient measurements should be performed in order to avoid large errors in particle concentration measurement. In a mixture of solids, i.e. cell debris and IBs, optical density measurements cannot be used to size one type of particles when used in combination with CSA, because the method lacks the ability to discriminate between particles. Thus, optical density measurements are only useful in combination with CSA for the size determination of particles that are larger than the wavelength of light in a solution without other solids present.

2.3. Muramic acid detection

In biological samples lactic acid, muramic acid and glyceraldehyde may be present. These substances contain a lactic acid moiety that can be quantified with the method of Barker and Summerson [22]. In order to discriminate between muramic acid present in peptidoglycan (cell debris) and the lactic acid moieties in solution, a number of methods are available. A simple solution is the analysis of acid treated samples, where peptidoglycan is hydrolysed and untreated samples, where peptidoglycan is still intact. Subtraction of the concentration measured in the untreated samples from the concentration measured in the acid treated samples yields the muramic acid concentration originating

from peptidoglycan [9]. A large disadvantage of this method is that in subsequent steps of the analysis peptidoglycan may be hydrolysed, which causes the measured concentration in the unhydrolysed samples to be higher than the actual concentration. An alternative to this method is to wash the solid cell debris with liquid to remove all dissolved components before peptidoglycan hydrolysis. However, a disadvantage of this method is the loss of peptidoglycan during the washing procedure. A much easier and more reliable third method is the analysis of the liquid phase without particles and the concentrate separately. By subtracting the concentration in the supernatant from the concentration in the concentrate, accurate quantification of peptidoglycan-associated muramic acid is possible. However, this method is only useful for cell debris since the dissolved lactic acid concentration in whole cells may differ from the concentration in the extracellular liquid. These intracellular lactic acid components have to be washed out of the cells before discrimination between peptidoglycan-associated muramic acid and lactic acid moieties in solution is possible. In this paper we work with whole cells, because whole cells are more defined than cell debris particles. Washing steps are thus required for accurate quantification of peptidoglycan-associated muramic acid.

The influence of interfering components present in biological samples on the spectrophotometric muramic acid assay has been studied thoroughly by Barker and Summerson [22] and Taylor [25]. Barker and Summerson suggested two simple washing steps that should remove the major part of these components before the actual colouring reaction is performed. These washing steps consist of protein precipitation with trichloroacetic acid “wash 1”, and an interfering component absorption step with copper and calcium “wash 2”.

Each handling step adds inaccuracy, and subtraction of concentrations increases relative errors. The beauty of SDS–PAGE on the concentrate is that minimal handling is required if you completely treat the pellet. Since the supernatant can be mixed homogeneously just by shaking, SDS–PAGE analysis is very easy without the need for additional steps. A disadvantage of SDS–PAGE, on the other hand, is that chemical treatment or storage of cells and cell debris may affect proteins, which may lead to destruction of membrane-associated proteins or their release from the cell wall, causing errors in cell debris quantification and PSD determination.

2.4. Acetaldehyde detection

A simple alternative to the spectrophotometric assay employs acetaldehyde quantification by GC. This method circumvents the purification steps needed between peptidoglycan hydrolysis and the actual quantification analysis, since the GC column separates acetaldehyde from other components that are present. There is also an enzymatic acetaldehyde quantification kit (Boehringer Mannhein/R-Biopharm) that uses the oxidation of acetaldehyde to acetic acid coupled with the reaction of NAD^+ into NADH. These two methods are faster than the muramic acid assay of Barker and Summerson and the detection limits of all three methods are approximately the same (∼1 mg/l). One large disadvantage of GC detection and the enzymatic assay for acetaldehyde is interference of the assay by evaporation, oxidation and polymerisation of acetaldehyde [30].

3. Materials and methods

3.1. Fermentation

E. coli XA90 fermentations were performed in a 500 ml shake-flask on Luria medium (Miller’s modification) at 37 °C. The broth was used within 1 day. *P. putida* KT2442 was produced in a 10 l fermentor that was run in fed-batch mode at 30 °C as described by Weusthuis et al. [31]. The pH was maintained at 7.0 with a 25% ammonia solution, which also served as the main nitrogen source for the microorganism. Coconut oil free fatty acids (Vereenigde Oliefabrieken, Rotterdam, The Netherlands) were used as carbon source. The use of this carbon source forces the organism to produce inclusion bodies of medium-chain-length polyhydroxyalkanoate (mcl-PHA), which is a biodegradable polymer. After fermentation, the broth was stored at 4–8 °C for a maximum of 3 months.

3.2. Cumulative sedimentation analysis

In this work the cumulative sedimentation analysis procedure, as described by Wong et al. [21], was performed at 2862 × *g* in a MSE Minor ‘S’ centrifuge equipped with a swing-out rotor. The fermentation broth of *P. putida* was diluted with Milli-Q water to a dry mass of approximately 2.5 g/l. The fermentation broth of *E. coli* had a dry mass of approximately 1.7 g/l and was not diluted. Samples of 10 ml were centrifuged for the desired effective settling times. After centrifugation the supernatant (8 ml) and concentrate (2 ml) were separated and samples were taken for peptidoglycan and membrane-associated protein quantification. For calculation of the particle diameter Wong et al. [21] used a density of 1085 kg/m^3 for cell debris of *E. coli*. The density of *P. putida* cells was measured with Percoll density gradient marker beads (Amersham Biosciences). The density and viscosity of the liquid phase were measured with a DMA 48 density meter (PAAR) and a VT 550 viscosity meter (HAAKE).

3.3. SDS–PAGE

Samples were taken of the concentrate or the supernatant. The samples were centrifuged in an Eppendorf centrifuge at 25,000 × *g* to settle all protein marked cell debris particles with a diameter above 0.07 μm. The pellet was mixed with sample buffer (Invitrogen NuPAGE LDS) and boiled

for 10 min. A 12% Bis Tris gel (Invitrogen) was used in an Invitrogen Xcell surelock^{TM} mini-cell with MOPS running buffer and a broad range protein marker (Bio-Rad precision standards unstained: 250, 150, 100, 75, 50, 37, 25, 15, 10 kDa). The gel was stained with a 0.2% Coomassie blue solution for 2 h before destaining with a methanol (30%, v/v), acetic acid (10%, v/v) and Milli-Q water (60%, v/v) solution. After destaining the protein bands were analysed with densiometry (Kodak Scientific Imaging Systems 1D v. 3.5.4, Eastman Kodak Company).

3.4. Protein 50 Labchip®

Samples of the concentrate were treated with the Protein 50 Labchip® sample preparation method of Agilent Technologies. β-Mercaptoethanol was added to reduce the proteins in the samples. The samples were loaded onto the chip and the assay was performed in the Agilent 2100 Bioanalyser. In the assay a fluorescent dye is linked to SDS. Due to adsorption of SDS onto the proteins, the proteins are labelled with the fluorescent dye. Since fluorescence is measured in the effluent of the gel an accurate measure of the protein concentration is obtained. The area of the membrane-associated protein peaks in the electropherogram was used to calculate the sedimented protein mass fraction (F).

3.5. Muramic acid analysis

The concentrate phase was washed three times with a 20 mM TRIS 10 mM EDTA buffer of pH 8.2 by centrifugation and re-suspension to remove all muramic acid and lactic acid from the liquid phase. A calibration curve was made with lactic acid (Acros Organics) solutions with a concentration between 0 and 1000 μg lactic acid/ml Milli-Q water. One millilitre samples of the concentrate and the calibration curve were mixed with 2 ml 5 M sulphuric acid and heated for 2 h at 90–100 °C. After cooling down to room temperature 2 ml 10 M sodium hydroxide in water was added in order to lower the pH. Then 1 ml of 0.30 mg trichloroacetic acid/ml Milli-Q water was added to precipitate all proteins from the solution (wash 1). The mixture was then centrifuged for 15 min at 5610 × g in the MSE Minor 'S' centrifuge. Subsequently 2.5 ml of the supernatant was mixed with 0.5 ml 20% (w/w) copper sulphate pentahydrate in water and the volume was set to 5 ml with Milli-Q water. The pH was neutralised by adding 100 μl 25% (v/v) ammonium hydroxide. Approximately 0.5 g of calcium hydroxide was added and the mixture was shaken vigorously (wash 2). After at least 30 min with occasionally shaking the tubes were centrifuged for 15 min at 5610 × g.

The supernatant, now free of proteins and other interfering substances, was collected for the colorimetric muramic acid analysis as described by Taylor [25]. Three samples of the supernatant were diluted with 96% sulphuric acid at a volume ratio of 1:6. The samples were heated at 90–100 °C for at least 30 min in borosilicate tubes with a screw cap. After cooling down to room temperature, 50 μl of a 4% (w/w) copper sulphate pentahydrate solution in water was added to 3.5 ml of the samples. Then 100 μl of a 1.5% (w/w) p-phenylphenol solution in 95% ethanol was added and the liquid was immediately mixed well. After at least 30 min the absorbance was measured at 570 nm. The muramic acid analysis procedure was tested on pure muramic acid and peptidoglycan (Biochemika) and gave an accurate quantification of the compounds.

3.6. GC

Concentrate and/or supernatant samples were mixed with an equal volume of 5 M H_2SO_4 and heated at 90–100 °C for 3 h. The duration of the acid reaction was assumed to be optimal after 3 h, since the acetaldehyde quantity remained constant after this reaction time. Subsequently, 10 M NaOH was added to neutralise pH and 0.5 μl samples were injected onto a HP cross-linked polyethylene glycol innowax column (15 m × 0.53 mm with a film thickness of 1.0 μm) of Agilent Technologies (The Netherlands) at 70 °C that was linked to a flame-ionisation detector. Helium was used as carrier gas with an inlet pressure of 85 kPa.

4. Results and discussion

The enzymatic acetaldehyde assay could not be used for cell debris quantification due to interfering components that could not be removed in washing steps 1 and 2. This conclusion is based on the fact that mixing of a standard solution of acetaldehyde with the same volume of the samples did not yield the concentration expected on basis of the concentrations measured in the separate solutions.

The acetaldehyde concentrations measured with the GC analysis did not give an accurate measure for the muramic acid contents of the samples when compared to the other methods. There are two reactions that could interfere with the acetaldehyde measurement, i.e. oxidation of acetaldehyde into acetic acid [30] and polymerisation of acetaldehyde under influence of sulphuric acid [32]. Our hypothesis is that acetaldehyde is polymerised during the acid treatment of peptidoglycan, since acetic acid concentrations were below 10% (mol/mol) of the acetaldehyde concentration in all samples. In the MA assay, acetaldehyde and its polymer react with p-phenyl phenol and copper ions into chromagen, while in the GC method the polymer remains in the liner of the GC or is broken up into other components unidentified, which gives large errors in acetaldehyde quantification.

In Fig. 3 the results of OD600 measurements, SDS–PAGE and the spectrophotometric muramic acid detection method for *E. coli* (A) and *P. putida* (B) are shown. The results are compared by plotting the mass fraction of particles that settled from the settling zone (F) versus the logarithm of the effective settling time ($\ln t_e$). For both organisms the results of the analysis methods are within experimental accuracy. The

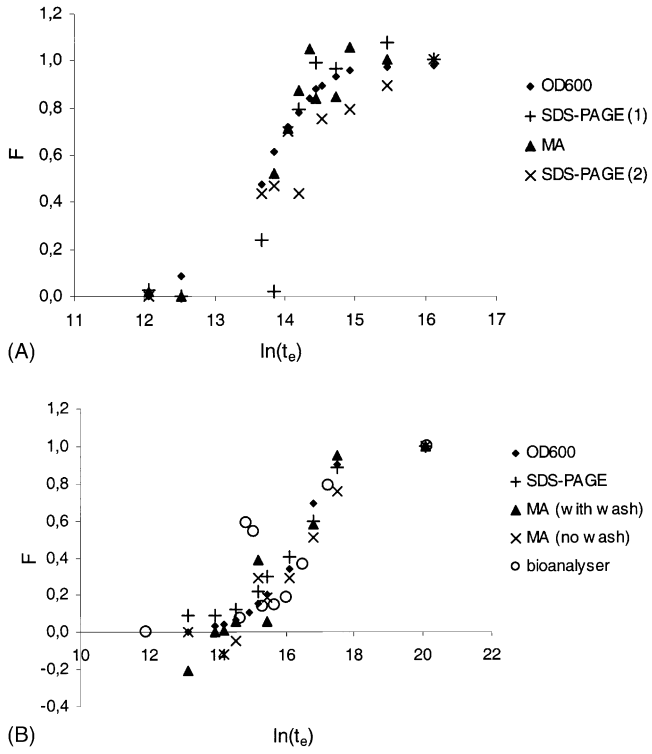

Fig. 3. Results of CSA in combination with SDS–PAGE analysis of membrane-associated proteins, OD measurements and colorimetric MA quantification for *E. coli* (A) and *P. putida* (B). In both graphs t_e is in seconds.

data for SDS–PAGE are based on the membrane-associated proteins of approximately 37 kDa for *E. coli* [33] and 19 kDa for *P. putida* [34]. A typical gel obtained with SDS–PAGE is shown in Fig. 4.

SDS–PAGE analysis for *P. putida* was very accurate in comparison to the data for *E. coli*. The large difference was probably due to the lower protein loading in the case of *E. coli*, which makes the interpretation with densiometry more difficult because of lower absolute band-intensity differences. The fact that the SDS–PAGE analysis gives accurate results for *P. putida* and *E. coli* indicates that the analysis method may be applicable to a broader range of microorganisms that have a similar membrane-associated protein content.

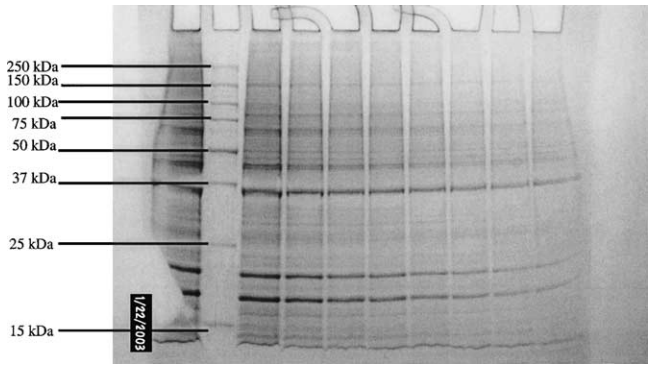

Fig. 4. SDS–PAGE gel for *P. putida*. Lane 2: marker, lanes 1, 3–10: concentrate fractions as effective settling time is decreased from 5.15×10^8 to 0 s at $2862 \times g$.

The Protein 50 Labchip® assay of the membrane-associated proteins of *P. putida* was very accurate. Two data points, at $\ln(t_e)$ of 14.9 and 15.1, respectively, gave a settled particle mass fraction that was much too high. This inaccuracy can be ascribed to the opening of the Eppendorf vessels during sample preparation, which resulted in evaporation of liquid, and therefore, caused an increase in protein concentration. A problem with the use of the Protein 50 Labchip® is the shift in peak elution time for different samples, which makes it difficult to obtain the size of the eluted proteins. The method corrects for this shift in elution time by including two internal standards of 3.5 and 53 kDa, respectively. However, if a sample includes proteins that overlap with the internal standard proteins, which often is the case with cell lysates, it becomes very difficult to determine the exact size of the eluted proteins. In our case the membrane-associated proteins could be located exactly by comparing the results standard SDS–PAGE with the results from the bioanalyser.

The data for the spectrophotometric MA assay are based on the average of three measurements of the supernatant of the copper/calcium adsorption step (wash 2). The data-points with more than 15% (standard deviation/average × 100%) inaccuracy are omitted. These inaccuracies are mainly due to Cu^{2+}/Ca^{2+} precipitates of wash 2 that float on top of the supernatant. When a sample is taken for the colouring reaction some of these particles may be taken along. Addition of sulphuric acid causes the particles to dissolve, which gives interference with the absorption measurement since the particles contain interfering components. To solve this problem the supernatant should be centrifuged and transferred to a clean tube. The MA assay was also performed without washing steps 1 and 2. The measured concentrations in these assays were much lower than the concentrations when the washing steps were included. This difference is apparently caused by the presence of interfering components that, for instance, could suppress light absorption by chromagen. When the washing steps are omitted these components stay in the mixture and interfere with the assay. Nevertheless the CSA results were very accurate. This is probably due to the relation between the interfering component concentration and the peptidoglycan concentration. A higher cell debris concentration gives a higher MA concentration but also a proportionally higher interfering component concentration that quenches the light absorption. The obtained muramic acid concentrations thus give an accurate measurement for the CSA procedure due to the use of relative concentrations, but omission of the washing steps does not give a quantitative measure for peptidoglycan and cell debris.

In order to get an estimate of the particle size we need to know the density difference between the fluid and the cells. *P. putida* cells have a density between 1040 and 1070 kg/m^3, which was measured with density gradient centrifugation. Olbrich [35] reported a density for *E. coli* cell debris in the

range of 1061–1090 kg/m^3. Wong et al. [21] used a density of 1085 kg/m^3 for cell debris, since no accurate method for cell debris density determination exists. The difference in densities for *E. coli* debris and *P. putida* whole cells could be caused by the surface/volume ratio of the cells. The *P. putida* cells contain approximately 30% (w/w) PHA inclusion bodies (measured with GC method of de Rijk et al. [36]) that have a density close to the density of water [37]. This significantly lowers the average density of the whole cells compared to cell debris, which mainly consists of cell wall components. *E. coli* cells contain water (and organelles), which will also lower the density of the cells compared to cell debris. The uncertainty in cell density does not have a large influence on the predicted Stokes diameter due to the quadratic dependence of the Stokes diameter in Eq. (1). When the density of whole cells is assumed to be 1085 kg/m^3, the Stokes diameter can be calculated from Eq. (1). Also the oversized settled particle mass fraction, W, can be obtained by making a fit of F versus $\ln(t_e)$ with SigmaPlot® 8.2 from the graphs in Fig. 3, which is used to calculate W with Eq. (2). The result of this operation is shown in Fig. 5 for *P. putida* (A) and *E. coli* (B). It is clear that this action smoothens the original F versus $\ln(t_e)$ data and is thus less sensitive to errors in the original measurements. The graphs show that the Stokes diameter of *P. putida* cells is between 0.09 and 0.8 μm with a median diameter of 0.40 μm, while that of *E. coli* is between 0.4 and 1.6 μm with a median of 1.22 μm. The difference in PSD for *E. coli* and *P. putida* can be due to the longer storage time of *P. putida* cells or the dilution of these cells in Milli-Q water (osmotic shock). Storage and osmotic shock may result in cell breakage, autolysis, degradation or permeabilisation, yielding smaller Stokes diameters.

In theory, optical density should give an accurate estimate of the actual particle concentration for all particles that have a linear relation between the extinction coefficient and the particle diameter. Optical density measurements were performed at 600 nm; hence particles with a size near or below 600 nm could give problems in the optical density measurement. This indicates that for *E. coli* there should not be a problem with the optical density measurement at high effective settling times (t_e) values, while for *P. putida* we may expect deviations from the actual concentration. These deviations are not clearly visible in the measurements, because of the low number of data-points at high effective settling times (only three data-points with an estimated particle size below 0.60 μm). When working with particles with a diameter smaller than that of whole cells, deviation from actual concentrations will occur when optical density is used for particle quantification without extinction coefficient measurements.

The PSDs obtained with different analysis methods, as shown in Fig. 5, have a small deviation from the PSD obtained with optical density measurements, which is expected to represent the most accurate PSD in this case. The SDS–PAGE analysis has the largest deviation. This is probably due to the low number of data-points in the F versus $\ln(t_e)$ plot in the region with the steepest slope, which makes it difficult to obtain a representative fit of the F versus $\ln(t_e)$ curve. The same is true for the MA assay for *E. coli*.

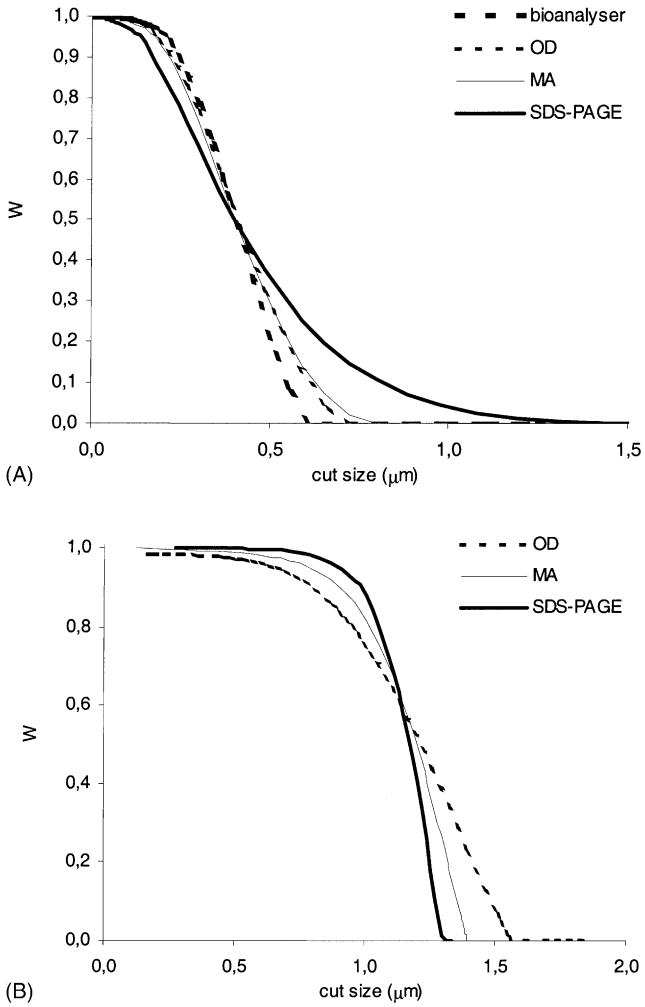

Fig. 5. Calculated values for W for SDS–PAGE analysis of membrane-associated proteins, OD measurements and colorimetric MA quantification for *P. putida* (A) and *E. coli* (B).

5. Conclusions

The GC method and the enzymatic assay of acetaldehyde were not useful for the quantification of muramic acid in our samples. The GC method was probably disturbed by a polymerisation reaction of acetaldehyde under influence of base or acid. The enzymatic assay on the other hand, was inaccurate due to interfering components that were not removed by wash 1 and wash 2.

SDS–PAGE analysis of membrane-associated proteins, the Protein 50 Labchip® analysis and the spectrophotometric muramic acid assay can be used for quantification of cell debris throughout a process and the determination of cell debris PSD when used in combination with CSA. The

Protein 50 Labchip® analysis is a very fast and accurate method. However, size determination of the eluted proteins may become a problem when the sample includes proteins with a size that overlaps with the internal standards. The results of the bioanalyser are more accurate than the results from standard SDS–PAGE with densiometry due to fluorescence detection, which gives a more reliable quantification of proteins than densiometry. Fluorescence detection is also possible in combination with standard SDS–PAGE, but since the bioanalyser assay is fully automated it is a more favourable method. Lundebye et al. [38] reported the semi-quantification of stress protein levels in organisms with western blotting in combination with densiometry. The variability of samples on one gel was in the range of 5–12% when raw absorbance data were used. The variability was much lower, in the range of 3.4–6.8%, when a calibration curve was run on the same gel. Taylor [25] showed that the spectrophotometric muramic acid assay has a variability less than 3.3%. Taylor did not use washing steps 1 and 2 for removal of interfering components. These washing steps cause the variability to increase with approximately 0.5%, which is comparable to SDS–PAGE in combination with densiometry. The buffer wash to remove all dissolved components from the cells, which is only required when working with whole cells, introduces a larger error in the analysis. Since the analysis is intended for the quantification of cell debris instead of whole cells (OD measurements sufficient in that case) this buffer wash can be omitted in the future.

Peptidoglycan-based detection methods in general have an advantage over the membrane-protein detection methods, because no information is needed about the size of the membrane-associated proteins. Also peptidoglycan detection methods can be easily applied to mixed cultures when the cell walls have the same peptidoglycan content, while SDS–PAGE requires the analysis of membrane proteins of each organism separately. In some cases proteins may be affected due to sample storage or addition of chemicals throughout a process. This may lead to errors in cell debris quantification and PSD determination with the protein-based assays. Peptidoglycan-based assays are a good alternative in these cases.

6. Nomenclature

A_λ	light absorbance at wavelength λ (A)
c	particle concentration (#/m^3)
D	Stokes diameter (m)
F	mass fraction of particles that settled to the settling zone (kg/kg)
g	gravitational acceleration (m/s^2)
H	settling height (m)
l	length of path through solution (m)
t_e	effective settling time (s)
v_g	settling velocity (m/s)
W	oversized settled particle mass fraction (kg/kg)
$\Delta\rho$	density difference between particles and liquid phase (kg/m^3)
ε	extinction coefficient
η	liquid viscosity (Pa s)

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