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(54) Titre : PROCEDE DE TRAITEMENT DE BIOMASSE POUR LA PRODUCTION DE LYSAT CELLULAIRE CONTENANT DE L'ADN PLASMIDIQUE

(54) Title: METHOD FOR TREATING BIOMASS FOR PRODUCING CELL LYSATE CONTAINING PLASMID DNA

(57) Abrégé/Abstract:
The invention relates to a method for the integrated treatment of biomass from a cell culture process for producing clear cell lysate containing plasmid DNA. According to the invention, in a first step filtering occurs in a filter in the presence of a filtering agent. In a second step, the biomass contained in the filter cake which is obtained is thermally decomposed and the cell lysate is filtered in another step. The obtained cell lysate containing plasmid DNA is characterised by a clarity of OD_{600} of at a maximum of 0.05E/cm and which is suitable for cloning, transformation, transfection, microinjection in cells, gene therapy, DNA vaccination and/or for polymerase chain reaction (PCR).

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(54) Title: METHOD FOR TREATING BIOMASS FOR PRODUCING CELL LYSATE CONTAINING PLASMID DNA

(54) Bezeichnung: VERFAHREN ZUR AUFBEREITUNG VON BIOMASSE ZUR HERSTELLUNG VON PLASMID DNA HALTIGEM ZELLLYSAT

(57) Abstract: The invention relates to a method for the integrated treatment of biomass from a cell culture process for producing clear cell lysate containing plasmid DNA. According to the invention, in a first step filtering occurs in a filter in the presence of a filtering agent. In a second step, the biomass contained in the filter cake which is obtained is thermally decomposed and the cell lysate is filtered in another step. The obtained cell lysate containing plasmid DNA is characterised by a clarity of OD_{600} of at a maximum of 0.05E/cm and which is suitable for cloning, transformation, transfection, microinjection in cells, gene therapy, DNA vaccination and/or for polymerase chain reaction (PCR).

(57) Zusammenfassung: In einem Verfahren zur integrierten Aufbereitung von Biomasse aus einem Zellkulturprozess zur Herstellung von klarem, Plasmid DNA haltigem Zelllysat wird jeweils in Gegenwart eines Filterhilfsmittels in einer ersten Stufe in einem Filter filtriert, in einer zweiten Stufe die im erhaltenen Filterkuchen enthaltende Biomasse thermisch aufugeschlossen und in einer weiteren Stufe das Zelllysat filtriert. Das erhaltene Plasmid DNA haltige Zelllysat zeichnet sich durch eine Klarheit OD_{600} von höchstens 0.05E/cm aus und ist zum Klonieren, zur Transformation, zur Transfektion, zur Mikroinjektion in Zellen, zur Gentherapie, zur DNA Vakzinierung und/oder zur Polymerasen-Ketten-Reaktion (PCR) geeignet.

WO 02/057446 A3

Method for treating biomass for producing cell lysate containing plasmid DNA

The present invention relates to a method for the integrated treatment of biomass from a cell culture process for producing clear cell lysate containing plasmid DNA, and a plasmid DNA cell lysate produced by the method.

Treatment is to be understood as meaning a conditioning method for providing clear cell lysate containing plasmid DNA. An integrated method is to be understood as meaning a method in which the individual steps of the method are contiguous with one another so that the product stream is conveyed virtually continuously. The basically multistage method can be carried out continuously or batchwise.

The separation of biological material from a cell culture process, the digestion of the biological material and the production of a clear cell lysate containing plasmid DNA is a customary method in the area of molecular biology. In the known methods, biological material, for example comprising E. coli bacteria cells, is separated from the culture supernatant, resuspended and then digested. Separating off the solid constituents gives a clear cell lysate which contains the plasmid DNA in addition to genomic DNA, RNA, proteins and endotoxins.

It is known that biological materials can be separated from the cell culture process by batchwise or continuous centrifuging. The separation of baker's

yeast cells by filtration over a bed of filtering agent is described in GB-A-1082862. Said patent also discloses the separation of cell residues from a yeast autolysate by means of a filtering agent.

The customary methods for cell digestion are the known alkaline lysis and thermal lysis. In order to improve the cell digestion, enzymes, for example lysozyme, and/or detergents are frequently added to the cell suspension. In the alkaline lysis method, a precipitate which substantially contains the cell debris and parts of the genomic DNA and of the protein is obtained after the cell digestion at pH 12 by addition of sodium hydroxide solution and sodium dodecylsulphate and subsequent neutralization with a high molecular weight acetate buffer. The complete isolation of this precipitate can be achieved only by thorough centrifuging. A centrifugal acceleration of 12000 g is not sufficient for this purpose (I. Feliciello et al., Anal. Biochem. 212 (1993) 394-401). The precipitate can be very substantially isolated only at a centrifugal acceleration of 26000 g for 30 minutes at 4°C. On the other hand, the filtration of this precipitate is associated with low process rates and large losses of plasmid DNA, even with the aid of very fine filtering agents or in combination with a flotation step. The adsorption of plasmid DNA on the surface of the filtering agent contributes considerably to the losses. In particular, plasmid DNA binds very rapidly and strongly to such mineral surfaces if the concentration of divalent cations exceeds 0.1 mmol or the monovalent cations exceed 20 mmol in the case of potassium or 50 mmol in the case of sodium (G. Romanowski et al., Appl.

Environ. Microbiol. 57 (1991) 1057-1061).

High molecular weight nucleic acids are sensitive to shear forces. Strong shear forces can lead to irreversible damage to nucleic acids, in particular to breaks in the strand. For this reason, mechanical digestion methods are seldom used for nucleic acid treatment (A. Carlson et al., Biotechnol. Bioeng. 48 (1995) 303-315). It is also known that, in industrial centrifuges with continuous introduction of liquid, shearing stresses at the rotor entrance act on the nucleic acid, inevitably leading to strand failures.

WO 96/36706 describes a process in which microorganisms are digested in the presence of detergents (Triton®) and by heating to 70-100°C in a throughflow heat exchanger. This process is a combination of thermolysis and detergence (optionally plus enzyme). Without Lysozyme, a temperature treatment for 30 seconds is described; with Lysozyme, one of 6 seconds. In the digestion of cells by heating, solid compounds are formed from debris, genomic DNA and proteins. Besides, the heat treatment may be supposed to accomplish a very far-reaching denaturing of enzymes decomposing DNA, called DNases. A clear cell lysate is obtained after batch centrifuging. In this process, only the cell digestion is carried out continuously; the separation of the solid constituents is accomplished batchwise by centrifuging. To obtain the final clear cell lysate, it was necessary to perform a filtration on a membrane filter following the centrifuging.

According to WO 92/07863, there are a known process and device for isolating nucleic acids from cell suspensions. Here, the cells are immobilized in the form of a layer in the cavities of a prepared porous matrix. This is accomplished by depth filtration in the matrix, the cavity size being on the order of magnitude of the cells and the matrix surface having ion-exchanger properties. Owing to the ion-exchanger properties, DNA absorbs at the matrix surface. Absorption of DNA is not a subject of the invention. The known process yields a purified DNA, but not a clear, plasmid DNA.

EP-A-0,814,156 describes a process for purifying DNA. The kieselguhrs are not specified in detail. The lysis takes place by the alkaline process. No thermal lysis is described.

The prior art thus discloses neither methods which make it possible to work up large amounts of biomass in an integrated manner nor methods by means of which large amounts of clear cell lysates containing plasmid DNA can be provided by an integrated procedure.

There is a need for an efficient conditioning method for providing clear cell lysate containing plasmid DNA from a cell culture process which permits plasmid DNA purification.

It is therefore the object of the present invention to provide an integrated working-up method which makes it possible to provide large amounts of clear cell lysates

with high plasmid DNA yield from a biomass in an efficient and controlled manner while avoiding the disadvantages of the alkaline lysis method and of centrifuging.

This object is achieved, according to the invention, if in each case filtration is effected in a first stage in a filter in the presence of a filtering agent, the biomass contained in the filter cake is thermally digested in a second stage and the cell lysate is filtered in a further stage.

It has now been found that any volume from 50 ml to 10000 l of biomass can be rapidly processed to give clear cell lysate containing the plasmid DNA by the method of the present invention. Particularly advantageous is the fact that, in the method of the present invention, the applied excess pressure during filtration provides a check with regard to the shear forces acting on the nucleic acids. In particular, after separation by gel electrophoresis, a cell lysate produced according to the invention clearly shows the bands for plasmid DNA, in addition to the bands for genomic DNA and RNA, without detectable damage to the nucleic acids due to breaks in the strand. Moreover, the cell lysate obtained is freed from unpleasant odours by the heat treatment.

It is expedient that the filtering agent is present during the digestion. This has the advantage that the filtering agent required for the subsequent clarifying filtration of the cell lysate is already present. The continuous use of the same filtering agent over all

steps (isolation, digestion, clarification) is also efficient in terms of process engineering and therefore economical. It is particularly advantageous if, instead of suspending the filter cake for the lysis, combined thermal digestion and clarifying filtration are carried out directly in the existing filter cake (for example by pumping through hot lysis buffer) and thus very clear cell lysate can be obtained directly. Consequently, the entire method is more efficient and hence economically more advantageous as a result of the reduction of the number of individual steps.

The choice of the filtering agent is of decisive importance. The filtering agent must be inert in order to minimize adsorption effects. It has proved expedient to use, as the filtering agent, calcined kieselguhrs having an SiO_2 content of at least 90% by weight and additionally having the following properties:

- Wet density: 0.2 - 0.4 g/cm^3
- Permeability: 0.02 - 2 Darcy
- BET surface area: 2 - 20 m^2/g
- Geometrical surface area: 0.25 - 0.65 m^2/g
- Particle size: 2.5 - 8.0 µm
- Particle size retention (99%): 0.1 - 2 µm

This has the advantage that very clear filtrate having a sufficiently high filtration flow rate at moderate differential pressure is obtained in one filtration step. In addition to this combination of efficiency and effectiveness, the use of highly pure filtering agents has the advantage that their surfaces are standardized and hence their properties can be controlled.

It is also expedient that the same filtering agent is used in all stages. This has the advantage that the individual steps of the method are contiguous with one another so that the product stream is conveyed virtually continuously.

The amount of filtering agent, based on the solids content of the solution to be filtered, the filtration area and the level of the maximum excess filtration pressure to be applied can only be determined empirically.

It has proved particularly expedient to carry out the digestion thermally. This has the advantage that the digestion can be carried out under mild conditions, for example in the physiological pH range, under very readily controllable and reproducible conditions. The process rate can be considerably increased if a flow-through heat exchanger is used instead of the batchwise heating of the cell suspension. It may also be assumed that a part of the DNA-degrading enzyme is deactivated on heating. In comparison with the alkaline lysis, the odour annoyance in thermal lysis is substantially lower.

The digestion is carried out at temperatures between 70°C and 90°C, preferably at 70°C to 85°C, in particular at 80°C. At a temperature of less than 70°C, the thermal digestion is incomplete; at a temperature above 90°C, plasmid DNA melts. The duration of the thermal treatment can only be determined empirically. It is 30 seconds to a few minutes.

The digestion must be carried out at a pH between 7 and 10. In this pH range, the adsorption losses of plasmid DNA are minimal and the activity of lysozyme is optimal. Moreover, plasmid DNA is already present in the physiological pH range in the clear cell lysate obtained.

It is expedient to carry out the digestion in the presence of exclusively monovalent cations. This has the advantage that losses in the final filtration to give the clear plasmid DNA lysate owing to adsorption on the surface of the filtering agent are minimal. Polyvalent cations which are liberated during the cell digestion are masked by complexing agents.

The maximum concentration of cations should be not more than 20 mmol for K^+, not more than 50 mmol for Na^+ or not more than 150 mmol for NH_4^+. When these maximum concentrations are exceeded, losses of plasmid DNA occur owing to adsorption on the surface of the filtering agent. If a plurality of cation species are present, the additive nature of the effect must be taken into account when specifying the permissible total concentration.

Clear cell lysate containing plasmid DNA is distinguished by a clarity OD_{600} of not more than 0.05 U/cm, corresponding to a decrease in the turbidity of at least 99%. The resulting clear cell lysate with the plasmid DNA can be used for cloning, for transformation, for transfection, for microinjection into cells, for gene therapy, for DNA vaccination and/or for polymerase chain reaction (PCR).

The invention is to be described in more detail with reference to examples.

Example 1

Plasmid DNA pHEN1 (4618 bp) in the E. coli strain TG1 was multiplied in 2TY medium at 37°C for 16 hours in a shaken flask. 15, 30 or 60 g/l of CelpureP300® (trade mark of World Minerals Inc.) were added to three E. coli suspensions of 125 ml each (OD_{600} = 6) and stirring was carried out continuously at 20°C. Each suspension was then transferred to a suction filter (10 cm^2 filter area, 200 ml capacity) in which a prefilter cake of 2 kg/m^2 of CelpureP300® had been built up beforehand over a filter medium comprising polypropylene monofilament (pore size 10 µm). The filtration was carried out at an excess pressure of 0.5 bar and 20°C.

In all cases, a clear filtrate was obtained. The highest mean filtration flow rate (2.8 m^3/m^2h) was achieved with 30 g/l of CelpureP300®. The results are summarized in Table 1.

Table 1

CelpureP300®(*) in g/l	Mean filtration flow rate (m^3/m^2h)	OD_{600} of the filtrate (U/cm)	Decrease in turbidity in %
60	1.8	0.04	99
30	2.8	0.05	99
15	2.4	0.03	99

(*) Properties: Silica content: 98.65%; wet density: 0.26 g/cm^3.

Permeability: 0.15 - 0.30 Darcy; BET surface area: 4 - 6 m^2/g; geometrical surface area: 0.62 m^2/g; particle size retention (99%): 0.6 - 0.75 µm.

Example 2:

Lysis buffer A:

150 mmol of Tris·HCl, 25 mmol of Na_2EDTA, 8% by weight of sucrose (pH 9)

Lysis buffer B:

As for lysis buffer A, but additionally 2% of TritonX-100® (trade mark of Röhm und Haas)

Two filter cakes from Example 1 with 30 g/l of CelpureP300® were each resuspended in 190 ml of lysis buffer A or B. 500 U/ml of lysozyme were added in each case and the suspensions were heated to 80°C for 30 seconds with continuous stirring. The still hot suspensions were transferred to a suction filter in which a prefilter cake of 2 kg/m^2 of CelpureP300® (incubated for 15 minutes in the corresponding lysis buffer) had been built up over a filter medium comprising polypropylene monofilament (pore size

10 µm). The filtration was carried out at 0.5 bar excess pressure.

By means of the lysate buffer A, a clear cell lysate (OD_{600} = 0.02) with a mean filtration flow rate of 0.4 m^3/m^2h was obtained. In contrast, the mean filtration flow rate was reduced by 50% by the addition of Triton X-100® (lysis buffer B). A significant increase in the mean filtration flow rate to 1.5 m^3/m^2h in the lysis buffer A was achieved when the total amount of CelpureP300® had been adjusted to 100 g/l and when PVDF warp/PTFE weft monofilament (pore size 11.5 µm) had been used as the filter medium.

Example 3

3.7 ml of λ DNA (E. coli; 48.5 kb dsDNA; 0.51 mg/ml) were added to 25 ml of a buffer solution consisting of 250 mmol of Tris·HCl, 25 mmol of Na_2EDTA and 8% by weight of sucrose (pH 9.09). 0.75 g of CelpureP300® (30 g/l) was added and the suspension was shaken for 30 minutes at 20°C and 200 rpm.

UV absorption measurements at 260 nm and a PicoGreen® test (kit from Molecular Probes Inc.) showed no difference between the λ DNA concentration in the supernatant of the suspension and the concentration in the starting solution.

Example 4

E. coli cells DH5α with the plasmid DNA pEGFP-N1 were

divided into four equal parts and each part suspended in a buffer consisting of Tris·HCl and 10 mM EDTA. From one of these suspensions, the cells were digested using the Nucleobond® AX kit (trade mark of Macherey-Nagel) by the alkaline lysis method, and the plasmid DNA was isolated and purified. In the case of the other three suspensions, the cells were thermally digested for 5 minutes at 70°C/pH 9, 70°C/pH 10 and 60°C/pH 10, and the plasmid DNA was isolated and purified in each case using the same kit. The plasmid DNA quantification of the four samples by means of the PicoGreen test showed that the digestion by the alkaline lysis method and the thermal lysis at 70°C and pH 9 or pH 10 led to the same yields of the plasmid DNA, whereas the thermal digestion at 60°C and pH 10 gave only about 30% of the yield of the plasmid DNA obtained by the other three methods. From Example 3, it is known that no adsorption of the plasmid DNA on the surface of the filtering agent is to be expected under the thermal lysis conditions described.

Method of measurement:

Clarity measurement

The clarity of a filtrate (optical density, OD) was determined using a Lambda 20 UV/Vis spectrometer from Perkin-Elmer in the absorption mode at 600 nm and a wavelength of 1 cm against water as reference at room temperature.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for integrated preparation of biomass from a cell culture process for production of clear cell lysate containing plasmid DNA, characterized in that, in the presence of an inert filtering agent in each instance, filtration is performed in a filter in the first step, in a second step the biomass contained in a filter cake is thermally digested, and in a further stage the cell lysate is filtered.

2. The method according to claim 1, characterized in that the digestion is carried out at 70-90°C.

3. The method according to claim 1, characterized in that the thermal digestion is carried out for at least 30 seconds.

4. The method according to claim 1, characterized in that the inert filtering agent used is a kieselguhr having the following properties:

- Silicon dioxide proportion: ≥ 90%
- Wet density: 0.2-0.4 g/cm^3
- Permeability: 0.02-2 darcy
- BET surface area: 2-20 m^2/g
- Geometrical surface area: 0.25-0.65 m^2/g
- Particle size: 2.5-8.0 µm
- Particle size retention (99%): 0.1-2 µm.

5. The method according to any one of claims 1 to 4, characterized in that the same filtering agent is employed in all three stages.

6. The method according to any one of claims 1 to 5,

characterized in that the digestion is performed at a pH-value between 7 and 10.

7. The method according to any one of claims 1 to 6, characterized in that the digestion is performed in the presence of exclusively monovalent cations.

8. The method according to any one of claims 1 to 7, characterized in that the maximum concentration of cations is at most 20 mmol for K^+, at most 50 mmol for Na^+ or at most 150 mmol for NH_4^+.