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(71) Applicant(s)
Qiagen GmbH

(72) Inventor(s)
Torsten Schmidt; Karl Friehs; Erwin Flaschel; Martin Schleef

(74) Agent/Attorney
DAVIES COLLISON CAVE,1 Little Collins Street,MELBOURNE VIC 3000

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(71) **Applicant** *(for all designated States except US)*: QIAGEN GMBH [DE/DE]; Max-Volmer-Strasse 4, D-40724 Hilden (DE).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: SCHMIDT, Torsten [DE/DE]; Beethovenstrasse 7, D-32791 Lage (DE). FRIEHS, Karl [DE/DE]; Am Steinwedeler Wald 18, D-31319 Sehnde (DE). FLASCHEL, Erwin [DE/DE]; Telgter Strasse 2, D-33619 Bielefeld (DE). SCHLEEF, Martin [DE/DE]; Am Bergsiek 35, D-33739 Bielefeld (DE).

(74) **Agent:** VOSSIUS & PARTNER; Siebertstrasse 4, D-81675 München (DE).

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(54) **Title:** METHOD FOR THE ISOLATION OF CCC PLASMID DNA

(57) **Abstract**

The present invention relates to the production of biomass for the isolation of ccc plasmid DNA comprising culturing a bacterial transformant in a bioreactor containing an antibiotic-free batch medium under batch-conditions and, at the end of the batch phase, feeding under feed-back conditions the portion of a feed-back medium after the rise of DO above a threshold-set point. Said feed-back medium comprises besides a carbon source a magnesium salt, preferably in concentrations above 20 mM. Preferably, the bacterial transformant is harvested after the end of the culture and frozen or freeze-dried. Also preferred is that ccc plasmid DNA is, optionally directly, isolated after harvesting the bacteria.

METHOD FOR THE ISOLATION OF CCC PLASMID DNA

The present invention relates to the production of biomass for the isolation of ccc plasmid DNA comprising culturing a bacterial transformant in a bioreactor containing an antibiotic-free batch medium under batch-conditions and, at the end of the batch phase, feeding under feed-back conditions a portion of a feed medium after the rise of DO above a threshold-set point. Said feed medium comprises besides a carbon and a nitrogen source, a magnesium salt, preferably in concentrations above 20 mM. Preferably, the bacterial transformant is harvested after the end of the culture and frozen or freeze-dried. Also preferred is that ccc plasmid DNA is, optionally directly, isolated after harvesting the bacteria.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art of the present invention.

With the advent and progress of recombinant DNA technology into a variety of fields such as food stuff production and medical therapy, the desire for large quantities of highly pure DNA has constantly risen. Traditional methods of purifying genomic or plasmid DNA (see, e.g., Sambrook et al., "Molecular Cloning, A Laboratory Manual", CSH Press, 2nd edition, 1989, Cold Spring Harbor New York) usually require sophisticated methodology if the DNA is to be free from RNA and other contaminating organic compounds. In particular, methods for obtaining ccc plasmid DNA in pure form regularly suffer from the disadvantage that other plasmid topologies also produced have to be separated from the desired product. For example, Lahijani et al., Human Gene Therapy 7 (1996), 1971-1980 have reported that high yields of pBR322-derived plasmids intended for human gene therapy may be obtained when plasmids comprising a temperature-sensitive single-point mutation that affects the negative regulation of replication from the ColE1 origin of

replication are employed. Using this process, a yield of 2.2 g of plasmid DNA from a 10 liter-fed batch fermentation were reported. However, the bacterial transformants were grown in the presence of the antibiotic kanamycin which would render them unsuitable for registration and subsequent use in humans. Other approaches have tried to avoid the use of antibiotics; see, e.g., Chen et al., J. Industrial Microbiology and Biotechnology 18 (1997), 43-48. In this report, an automated fed-batch fermentation with feed-back controls based on dissolved oxygen and pH for the production of supercoiled plasmid DNA is disclosed. This DNA is suggested to be useful for DNA vaccines. However, the results reported, for example in Figure 4, do not support the suggested suitability of the plasmid DNA for vaccination purposes. This is due to the fact that besides ccc plasmid DNA a variety of other plasmid forms are produced under these conditions. Furthermore, this method leads to high contaminations with genomic DNA and, comparatively, only small plasmid amounts can be obtained.

Accordingly, ccc plasmid DNA produced by the prior art methods is unsuitable for a variety of purposes such as medical purposes due to the heterogeneity of the product obtained and/or due to the employment of antibiotics in the production process. The technical problem underlying the present invention was therefore to provide a method that overcomes these prior art difficulties and allows for the production of ccc DNA without the concomitant production of other plasmid forms and which is, moreover, suitable for medical purposes. The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Thus, the present invention relates to a method for the production of biomass for the isolation of ccc plasmid DNA comprising

(a) culturing a bacterial transformant in a bioreactor containing an antibiotic-free batch-medium comprising

(aa) a carbon source;

(ab) an inorganic salt mixture;

(ac) a nitrogen source;

under batch-culturing conditions;

(b) feeding under feed-back conditions to said culture of (a) at the end of the batch phase, after rising of DO above a threshold-set point, a portion of a feed-medium comprising

(ba) a carbon source; and

(bb) a magnesium salt; and

(c) allowing the bacterial transformant to metabolize said feed-medium.

The term "biomass", as used in the context of the present invention, relates to any biological material that is or arises from cells or organisms that are capable of reproduction.

The term "ccc plasmid DNA" refers to a plasmid isoform that is a circular plasmid which is typically but not necessarily underwound relative to a relaxed molecule. This results in a more compact conformity of the molecule which is described as a supercoiled covalently closed circle of the plasmid DNA. In E.coli cells, two enzymes regulate the supercoiling of DNA. The gyrase introduces negative superhelical turns into the molecule while the topoisomerase I relaxes the DNA by introducing single-strand breaks. It is most preferred in accordance with the method of the invention that ccc plasmid DNA in monomeric form is produced. Indeed, the method of the invention provides this particularly desired type of plasmid usually in an amount of more than 90% of overall plasmid production. Also useful, although less preferred, is the production of dimeric ccc plasmid DNA.

The term "batch-medium" refers to the medium used in batch cultivation, i.e., in discontinuous cultivation of bacterial transformants or other microorganisms. This discontinuous cultivation is characterized by a single inoculation into fresh medium (batch medium) at the start of the cultivation until nutrients and substrates have been exhausted.

The term "feed-back conditions" relates to the supplementation of medium concentrate during fed-batch cultivation depending on cultivation parameter(s) like, e.g., DO, pH, etc., which are correlated with the growth of the microorganism.

The term "threshold-set point" in the context of the present invention is intended to mean a defined value for a parameter that is monitored during fed-batch cultivation. In case of over-reaching or under-reaching of that value a monitor signal initiates the response in the regulation of the cultivation. The person skilled in the art is in the

position to determine such defined values on the basis of his common knowledge and the teachings of this invention; see, for example, example 1.

The term "DO" (concentration of dissolved oxygen) refers hereby to the amount of oxygen in a liquid in per cent of the saturation concentration.

Under the above-defined feed-batch conditions, the concentration of dissolved oxygen (DO) rises during cultivation of bacterial transformants after the consumption of nutrients such as contained in the feed-medium. Therefore, the invention relates in a preferred embodiment to a method wherein the feeding of a bacterial transformant culture comprises repeated feeding-cycles after each rising of the DO above a threshold-set point. It is well known to the person skilled in the art that feeding can be controlled and/or measured via other control parameters, like medium pH, specific growth rate of bacterial transformants, respiration coefficients or others. Nevertheless, all these parameters are interrelated and indicative of the DO. Therefore, irrespective of which parameter is actually employed as a read-out system, it allows a direct (or indirect) conclusion on the DO value. Accordingly, measurement of any of said parameters is covered by the invention as long as it allows a conclusion with respect to the rising of DO above a threshold-set point.

The term "allowing the bacterial transformant to metabolize said feed-medium" relates to the partial or complete metabolization of said feed-medium and preferably to the essentially complete metabolization of said feed medium.

In accordance with the present invention it has been found that the here disclosed method leads to the production of high plasmid concentrations and to plasmids with a DNA homogeneity of typically more than 90 % ccc monomers. The result is all the more surprising since ccc monomers of the indicated high homogeneity are obtained in the absence of selection pressure by antibiotics. The person skilled in the art is able to employ the here disclosed method for the production of ccc DNA over a broad range of types or sizes of plasmids. The method of the invention in addition allows the isolation of a larger quantity of the desired plasmid from bacterial cultures than was possible with prior art methods. This also leads to a significant cost reduction of the fermentation processes since smaller fermentors may be employed as compared to prior art processes if the same amount of plasmid is to be generated.

A further significant advantage of the method of the present invention for the production of biomass for the isolation of ccc plasmid DNA relates to the fact that the media are devoid of antibiotics. The obtained ccc DNA is thus definitively antibiotic-free and may, without time consuming and costly further elaborate purification schedules, be employed in medical therapy where antibiotic contaminations are to be avoided.

It is most preferred in the method of the present invention to employ batch-or feed media devoid of yeast extract or other complex amino acid sources derived from natural sources. This is because the cultivation in such fully synthetic media does not bear the danger of source related contamination and has the advantage of a higher reproducibility. Another preferred option would be to use semi-synthetic media, comprising, for example, yeast extracts, plant extracts, peptone supplements and others. These synthetic or semi-synthetic media may be employed in one or more steps of the culturing of the transformants. This also includes the pre-culturing step addressed below. It is also envisaged by the present invention to cultivate in/feed different types of media at different cultivation steps. Different media may also be fed when repeated feeding cycles are employed as discussed herein below. It is most preferred that these media are autoclavable. Magnesium salts should be autoclaved separately.

As has been outlined above, in a preferred embodiment of the method of the invention, step (b) comprises repeated feeding cycles after each rising of the DO above a threshold-set point. This embodiment of the invention is particularly advantageous since it allows the production of high yields of biomass which allows for the isolation of high amounts of ccc-plasmid DNA.

The bacterial transformant used in the method of the invention can be either a gram-negative or a gram-positive bacterium. Examples of gram-positive bacteria are bacteria of the genus *Bacillus*, for example *Bacillus subtilis*. In a preferred embodiment, the bacterial transformant is an *E.coli* cell.

Whereas the person skilled in the art is capable of devising or preparing a suitable carbon source, glycerol is preferably used as a carbon source in the method of the present invention.

A variety of well known and established nitrogen sources may be employed in the method of the invention. In a further preferred embodiment of the present invention, the nitrogen source employed is NH_3.

In a further preferred embodiment of the method of the invention the carbon source in the batch-medium is in a (initial final) concentration of ≤ 100 g/l.

In another preferred embodiment of the method of the invention the carbon source in the feed-medium is in a (initial final) concentration of ≤ 1000 g/l.

In yet another preferred embodiment of the method of the invention the nitrogen source is in a (initial final) concentration of ≤ 30%.

In a further preferred embodiment of the method of the invention the inorganic salt mixture comprises Na_2HPO_4 ≤ 6 g/l, KH_2PO_4 ≤ 3 g/l, NaCl ≤ 0,5 g/l, and citric acid•H_2O ≤ 1,5 g/l. This refers to the initial final concentration in the medium, i.e. the final concentration that is present in the medium at the onset of fermentation.

Most preferably, said inorganic salt mixture also comprises a magnesium salt, preferably $MgSO_4$, for example complexed with water. In this case, an initial concentration of smaller than or about 0.3 g/l is preferred. It is also desired that the magnesium salt is autoclaved separately.

In another preferred embodiment of the method of the invention in step (b) the magnesium salt concentration is in a range of 5-100 mM. This again refers to the initial final concentration in the medium.

In a particularly preferred embodiment of the method of the invention in step (b) the magnesium salt concentration is 80 mM. In accordance with the invention, it has

surprisingly been found that in particular rather high magnesium salt concentrations such as 80mM, preferrebly of $MgSO_4$, yield excellent results with regard to the homogeneity of the ccc plasmid monomers.

Magnesium salts that may be used in accordance with the present invention comprises $MgCl_2$, $Mg(NO_3)_2$, $MgSO_4$ or others. In a preferred embodiment of the method of the invention the magnesium salt is $MgSO_4$.

In another preferred embodiment of the invention a solution of trace elements is added in steps (a) and/or (b). Addition of trace elements may further enhance the plasmid yield.

In another embodiment of the method of the invention the solution of trace elements comprises each of the following compounds
$FeCl_3 \bullet 6\ H_2O$ in a final concentration of preferably ≤54.0 mg/l
$ZnSO_4 \bullet 7\ H_2O$ in a final concentration of preferably ≤13.8 mg/l
$MnSO_4 \bullet H_2O$ in a final concentration of preferably ≤18.5 mg/l
$CoSO_4 \bullet 7\ H_2O$ in a final concentration of preferably ≤5.6 mg/l
$CuCl_2$ in a final concentration of preferably ≤1.7 mg/l
H_3BO_3 in a final concentration of preferably ≤10 mg/l
$Na_2MoO4 \bullet 2\ H_2O$ in a final concentration of preferably ≤25 mg/l; and
citric acid in a final concentration of preferably ≤50 mg/l

Bacterial or prokaryotic growth media can advantageously be supplemented with an amino acid source. Therefore, in a further preferred embodiment of the method of the invention, the batch-medium comprises an amino acid source.

In another preferred embodiment of the method of the invention the feed-medium comprises an amino acid source. Amino acid sources are well known to the person skilled in the art and can comprise yeast extracts, plant extracts, peptone supplements and others (see Sambrook et al., loc. cit.).

In a further embodiment of the method of the invention the culturing of the bacterial transformant is carried out at a temperature range of 30°C to 42°C.

In a particularly preferred embodiment of the method of the invention the temperature range is about 35°C to 38°C.

In order to compensate auxotrophic requirements of bacterial transformants, it is preferred that in the method of the invention the batch-medium comprises a bacterial host strain specific supplement. A variety of specific supplements for different bacterial host strains has been described and is well known in the art, e.g., thiamine for bacterial transformants carrying a thiamine deficiency.

In a further preferred embodiment of the method of the invention the host cells are harvested, after step (c), from said cultures. Harvesting of bacterial transformants is one of the conventional methods in fermentation and molecular biology techniques. The harvest of transformants can comprise filtering, centrifugation or similar methods which are well known in the art.

In yet another preferred embodiment of the method of the invention, the host cells are, after step (c), subjected to a washing step before or after harvesting. These washing steps can be carried out in a solution that does not affect the integrity of the cells but removes culturing compounds from the cell.

In another preferred embodiment of the method of the invention, a further step comprises the freezing or freeze-drying of the transformants after step (c) or after the steps identified herein above in any of the further preferred embodiments. The embodiment is particularly useful if an immediate isolation of ccc plasmid is not desired. Frozen or freeze-dried cells may be conveniently be shipped or stored until further use.

In yet another preferred embodiment of the method of the invention a further step comprises the isolation of ccc DNA.

There are multiple ways to isolate ccc DNA which are well know to the person skilled in the art. These include CsCl gradient centrifugation and chromatography purification methods (see, e.g., Sambrook et al., "Molecular Cloning, A Laboratory Manual", CSH Press, 2nd edition, 1989, Cold Spring Harbor New York).

As stated herein above, isolated plasmid DNA consisting of more than 90 % ccc monomers may be obtained. The person skilled in the art, when employing the teachings of the present invention, is able to obtain ccc DNA in large quantities which fulfills the quality criteria of plasmid DNA for gene therapeutic and nucleic acid vaccination approaches (see Schorr et al., DNA Vaccines 772 (1995), 271-273) without subsequent exhaustive purification steps to get rid, for example, of antibiotics. Thus, it is possible to employ the obtained ccc DNA of the invention for nucleic acid vaccinations as described in the prior art, for example in Davis et al., Vaccine 12 (1994), 1503-1509, or for gene therapeutic strategies as disclosed in Cao et al., Human Gene Therapy 6 (1995), 1497-1501.

Furthermore, in another preferred embodiment, the invention relates to a method further comprising the step of (a') pre-culturing the bacterial transformant in an antibiotic-free medium.

In a particularly preferred embodiment of the method of the invention the bacterial transformant is in exponential growth phase after the end of said pre-culturing. The exponential growth phase may be assessed by conventional technology, for example, by determining the optical density of the culture broth.

The figures show

Figure 1: Time-course of dissolved oxygen, agitation speed and feed medium as monitored in a bioreactor during a DO feed-back controlled fed-batch cultivation. Shown is the cultivation of the plasmid pUK21CMVβ in *E coli* DH5α in a 30 L-bioreactor. Above a threshold set-point of 30 %, DO was controlled by increasing agitation speed. Below a threshold

set-point of 45 %, the DO was controlled by feeding a nutrient solution into the bioreactor.

Figure 2: Dry cell weight (DCW) and plasmid concentration during fed-batch cultivation of pUK21CMVβ in *E. coli* DH5α in semi-defined glycerol-yeast extract medium. The bacterial transformant was cultivated in a 30 l -bioreactor.

Figure 3: 0.8 % Agarose gel electrophoresis of plasmid DNA (each about 250 ng) at different cultivation times (10-40 h) of a 30 liter cultivation. Plasmid DNA was isolated from defined culture volumes using QIAGEN Miniprep kits.

Figure 4: Dry cell weight and plasmid concentration during fed-batch cultivation of *E. coli* DH5α containing pUK21CMVβ, using a synthetic glycerol medium. Fermentation was carried out in a 5 L-bioreactor.

Figure 5: 0.8 % Agarose gel electrophoresis of plasmid DNA (each about 250 ng) at different cultivation times (10-44 h) of a 7 liter cultivation. Plasmid DNA was isolated from defined culture volumes using QIAGEN Miniprep kits.

Figure 6: 0.8 % Agarose gel electrophoresis of pUT 649 plasmid DNA samples (200 ng/ 4 different isolates) at 41 h cultivation time, when cells were harvested. Plasmid DNA was independently isolated using QIAGEN Midiprep kits (Tip-100).

Figure 7: Final biomass of several cultivation conditions in yeast-extract (YE) media. Cultivations were performed as described in example 1 or described by Chen et al., J. Industrial Microbiology and Biotechnology 18 (1997), 43-48 or Lahijani et al., Human Gene Therapy 7 (1996), 1971-1980.

Figure 8: Final plasmid concentration of several cultivations in yeast-extract (YE) media. Cultivation was carried out as described in example 1 and compared to cultivations as described by Chen et al., J. Industrial Microbiology and Biotechnology 18 (1997), 43-48 or Lahijani et al., Human Gene Therapy 7 (1996), 1971-1980.

Figure 9: Plasmid yield per biomass of several cultivations in yeast-extract (YE) media at cell harvest. Cultivation was carried out as described in example 1 and compared to cultivations as described by Chen et al., J. Industrial Microbiology and Biotechnology 18 (1997), 43-48 or Lahijani et al., Human Gene Therapy 7 (1996), 1971-1980.

Figure 10: Restriction map of plasmid pUK21CMVβ

Figure 11: DNA sequence of plasmid pUK21CMVβ (SEQ ID NO: 2)

Figure 12: Restriction map of plasmid pUT 649

Figure 13: DNA sequence of plasmid pUT 649 (SEQ ID NO: 1)

The examples illustrate the invention.

Example 1: High-quality and high-quantity production of a 7.6 kbp ccc plasmid in glycerol/yeast media

The method for the production of biomass for the isolation of ccc plasmid DNA has been carried out by a feed-back controlled fed-batch cultivation of *Escherichia coli* DH5α containing the plasmid pUK21CMVβ (7612 bp) (Figures 10, 11) in a 30-L bioreactor (LAB 30 L, MBR, Switzerland) with a working volume of 23 l.

The semi-defined batch medium (15 l) consisted of 10 g/l glycerol, 5.0 g/l yeast extract, 6.0 g/l Na_2HPO4, 3.0 g/l KH_2HPO_4, 0.5 g/l NaCl, 1.5 g/l citric acid, 0.3 g/l

$MgSO_4 \bullet 7H_2O$, 5 mg/l thiamin hydrochloride and 10 ml/l trace element solution. Thiamin hydrochloride and the stock solution of trace elements (5.40 g/l $FeCl_3 \bullet H_2O$, 1.38 g/l $ZnSO_4 \bullet 7H_2O$, 1.85 g/l $MnSO_4 \bullet H_2O$, 0.56 g/l $CoSO_4 \bullet 7H_2O$, 0.17 g/l $CuCl_2$, 1.0 g/l H_3BO_3, 2.5 g/l $Na_2MoO_4 \bullet 2H_2O$ and 5.0 g/l citric acid) were sterilized separately. Before sterilization of the bioreactor (25 min at 121 °C), the pH of the medium was adjusted to 6.7 using NaOH.

Cultivation was carried out at 37 °C and 0.5 bar. Aeration was maintained at 30 l/min and pH was controlled by 10 % H_3PO_4 and/or 25 % NH_4OH. As antifoam reagent Pluronic® PE-8100 (BASF) was used. Minimum agitation speed was 100 rpm.

A frozen glycerol stock of *E. coli* DH5α including pUK21CMVβ was used to inoculate 200 ml of Luria-Bertani (LB) seed medium in a 1000 ml shake flask. The culture was incubated at 37 °C for 8 h on an orbital shaker at 180 rpm. After reaching a cell density of OD_{600} 0.3-0.5, a 50 ml aliquot of this pre-culture was transferred into the bioreactor.

During cultivation, dissolved oxygen (DO) was automatically kept at 30 % air saturation by increasing the agitation speed by 1 % of the previous agitation speed when dissolved oxygen concentration went below a threshold set-point of 30 %. At DO concentrations above 30 %, the agitation speed stayed constant. When the DO reached a threshold set-point of 45 % air saturation, a nutrient pump was automatically activated to feed a concentrated solution of 600 g/l glycerol, 90 g/l yeast extract and 20 g/l $MgSO_4 * 7 H_2O$ to the culture. The maximum flow rate of the nutrient pump was 50 ml/min. Feeding was interrupted when DO went below 45 %.

Every two hours cell growth was determined by measuring optical density at 600 nm and dry cell weight. Plasmid concentration was determined after isolating plasmid DNA from a defined pelleted culture volume using QIAGEN Miniprep kits (Tip-20) according to the instructions of the manufacturer. After digestion with restriction endonucleases EcoRI, quantification of these DNA samples was performed by capillary gel electrophoresis as described by Schmidt et al., J. Biotechnol 49 (1996), 219-229. In order to analyze the plasmid form distribution and as quality control, electrophoresis of all undigested samples was performed on 0.8 % agarose gels.

Figure 1 describes DO levels, agitation speed and feed solution values as monitored in the bioreactor during a DO feed-back controlled fed-batch cultivation. After 14 h, when the nutrients were depleted, DO concentration increased dramatically and the feeding loop started. During this fed-batch phase the DO concentration oscillated between the threshold set-point for feeding (45 %) and the threshold set-point for increasing agitation speed (30 %).

Figure 2 illustrates the formation of biomass (dry cell weight) and plasmid DNA during the cultivation. Cell growth occurred until 36 h cultivation time. A final biomass of 48 g/l dry cell weight (OD_{600} = 140) was reached. The plasmid concentration produced in this reactor was about 200 mg/l, which corresponds to 4,6 g plasmid DNA. The plasmid mass per cell weight was about 4,2 mg/g. Although no antibiotics for selection were used, plasmid concentration increased during the whole cultivation.

Agarose gel electrophoresis showed a high quality plasmid product during the whole cultivation (Figure 3). The isolated plasmid DNA consisted of more than 90 % ccc molecules and fulfilled the quality criteria of plasmid DNA for gene therapeutic and nucleic acid vaccination approaches (Schorr et al., DNA Vaccines 772 (1995), 271-273).

Example 2: High-quality production of ccc plasmid DNA in synthetic glycerol media

DO feed-back controlled fed-batch cultivation of *E. coli* DH5α containing pUK21CMVβ (7612 bp; SEQ ID NO: 2) was performed in a 7-L bioreactor (LAB 7 L, MBR, Switzerland) with 5,5 l working volume. Synthetic glycerol media were employed in this fermentation.

The defined batch medium (3,5 l) consisted of 20 g/l glycerol, 6.0 g/l Na_2HPO4, 3.0 g/l KH_2HPO_4, 0.5 g/l NaCl, 1.5 g/l citric acid, 0.3 g/l $MgSO_4 \bullet 7H_2O$, and 10 ml/l trace element solution, containing 5.40 g/l $FeCl_3 \bullet 6\ H_2O$, 1.38 g/l $ZnSO_4 \bullet 7H_2O$, 1.85 g/l $MnSO_4 \bullet H_2O$, 0.56 g/l $CoSO_4 \bullet 7\ H_2O$, 0.17 g/l $CuCl_2$, 1.0 g/l H_3BO_3, 2.5 g/l $Na_2MoO_4 \bullet 2\ H_2O$ and 5.0 g/l citric acid. 5 mg/l thiamine hydrochloride was sterilized

separately by filtration and was transferred into the bioreactor. Before sterilization of the bioreactor (25 min at 121 °C) the pH of the medium was adjusted to 6.7.

Cultivation was carried out at 37 °C and 0.5 bar. Aeration was maintained at 10 l/min and the pH was controlled by 10 % H_3PO_4 and/or 25 % NH_4OH. Antifoam reagent was Pluronic® PE-8100 (BASF). Minimum agitation speed was 150 rpm.

A frozen glycerol stock of *E. coli* DH5α including pUK21CMVβ was used to inoculate 55 ml LB seed medium in a 300 ml shake flask. The culture was incubated at 37 °C for 8 h on an orbital shaker at 180 rpm. After reaching a cell density of OD_{600} 0.3-0.5 50 ml of this pre-culture was transferred into the bioreactor.

During cultivation dissolved oxygen (DO) was automatically kept at 30 % air saturation by increasing the agitation speed by 1 % of the previous agitation speed, when dissolved oxygen concentration went below 30 %. At DO concentrations above 30 % air saturation, the agitation speed stayed constant. If DO was above 45 % air saturation, a nutrient pump was activated automatically to feed a concentrated solution of 1000 g/l glycerol and 20 g/l $MgSO_4 \bullet 7\ H_2O$ to the culture. The maximum flow rate of the nutrient pump was 30 ml/min. Feeding was interrupted when DO went below 45 %.

Every two hours cell growth was determined by measuring optical density at 600 nm and dry cell weight. Plasmid concentration was determined after isolating plasmid DNA from a defined pelleted culture volume using QIAGEN Miniprep kits (Tip-20) according to the instructions of the manufacturer. After digestion with restriction endonuclease EcoRI, quantification of these DNA samples was performed by capillary gel electrophoresis as described by Schmidt et al. 1996 (Schmidt et al., *J. Biotechnol.* 49 (1996), 219-229). In order to analyze the plasmid form distribution and as quality control, electrophoresis of all undigested samples was performed on 0.8 % agarose gels.

Figure 4 describes the formation of biomass and plasmid DNA during the cultivation. Cell growth occurred until 40 h cultivation time. A final biomass of 48 g/l dry cell weight (OD_{600} = 145) was reached using a synthetic medium. The produced plasmid concentration was about 100 mg/l, which corresponds to 550 mg plasmid DNA. The obtained plasmid mass per cell weight was about 2,1 mg/g.

As verified by agarose gel electrophoresis, plasmid product was obtained in a high quantity during the whole cultivation time (Figure 5). The isolated plasmid DNA was

obtained as ccc monomers. ccc dimers, which existed as concatemers, were found in small quantities. The DNA samples fulfilled the quality criteria of plasmid DNA for gene therapeutic and nucleic acid vaccination approaches.

Example 3: High-quality production of a plasmid for gene therapeutic approaches by high-salt density fermentation

A DO feed-back controlled fed-batch cultivation of *E. coli* DH5α containing pUT 649 (Figures 12, 13) (4618 bp; SEQ ID NO: 1) (The pUT 649 vector has been described in the Eurogentec-catalogue 1994 (Eurogentec, Liège, Belgium) under catalogue number VE-1134-a) was performed using the same glycerol yeast-extract media (7.5 l batch medium) as described in example 1. Similar cultivation conditions as described in example 1 were applied and the fermentation was performed in a bioreactor with 10 l working volume (BRAUN BioE). Aeration was 15 l/min. In the batch phase, the agitation speed was set to 800 rpm. In the fed-batch phase, this speed was raised by 100 rpm when the DO decreased below the threshold set-point of 30 %. Feed medium was pumped into the bioreactor (flow rate 10 ml/min), when the DO reached the threshold set-point 45 %.

In a 1 l shake flask, 100 ml batch medium was inoculated with 500 µl glycerol stock of *E. coli* DH5α containing pUT649. Incubation was carried out for 5 h at 37 °C on an orbital shaker. An 1.5 ml inoculum of this pre-culture was transferred into the bioreactor.

After 41 h cultivation time, when bacterial transformants reached the stationary phase, the final biomass yielded 60 g/l dry cell weight and 230 mg/l of plasmid. Plasmid DNA of more than 90 % ccc monomer plasmid DNA could be isolated (Fig. 6).

Example 4: Comparison between different batch and fedbatch culturing conditions

Batch cultivation in Luria-Bertani-(LB) medium (Sambrook et al., loc.cit.) in a 7 L-bioreactor, fed-batch cultivation as described in example 1 and fed-batch cultivations as disclosed in the state of the art were compared. Whereas Chen et al.

(J. Industrial Microbiology and Biotechnology 18 (1997), 43-48) used a dextrose-yeast extract medium in the batch phase and fed a glucose-yeast extract feed-medium, Lahijani et al. (Human Gene Therapy 7 (1996), 1971-1980) performed batch and fed-batch cultivations in antibiotic-containing glucose-yeast extract medium, employing a temperature-controllable point mutation in the bacterial transformant.

Figure 7 shows the final biomass when cells reached the stationary growth phase. Biomass yield in batch cultivations in LB medium or described by Lahijani et al. were low. Feeding of nutrient solutions, as described by Chen et al. or as described in the present invention, led to higher biomass yields.

As shown in Figure 8, in comparison to batch cultivations in LB medium, plasmid concentrations were increased by a factor 35-40 in fedbatch fermentations using glucose-yeast extract media, as described in Lahijani et al. or using glycerol-yeast extract media. as described in the present invention. However, fedbatch cultivations as carried out by Chen et al. led to lower plasmid concentrations.

Lahijani et al. used the antibiotic kanamycin during culturing and obtained a similar plasmid concentration as it was obtained with the method of the present invention, using antibiotic-free glycerol-yeast extract media. A comparison of biomass could not be carried out since Lahijani et al. did not disclose any figures concerning biomass obtained.
As depicted in Figure 9, fedbatch cultivation in glycerol-yeast extract medium led to higher plasmid yields per biomass as compared to batch conditions in LB medium. Highest plasmid yields per biomass could be obtained by batch cultivation in glucose yeast extract medium but the total plasmid yield was low.

The homogeneity and purity of isolated plasmid DNA was best under cultivation conditions performed according to the present invention (Fig. 3, 5, 6). Lahijani et al. reported that the isolated DNA contained large amounts of RNA and concatenated dimers, whereas Chen et al. obtained large amounts of contaminating genomic DNA. Hence, the removal of such contaminating non-ccc DNA, RNA or genomic

DNA requires additional processing steps, is time consuming and further reduces the yields of DNA isolated by prior art technologies.

In summary, the analysis of data allows the unambiguous conclusion that the method of the invention is superior of the prior art with regard to purity of the desired product, optionally or preferably in combination with the amount of desired end product obtained. The method of the invention is applicable to other plasmids and yields similar or the same advantageous results.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

AUSTRALIAN PATENT OFFICE

EDITORIAL NOTE - NO: 43658/99

Sequence listing pages 1-8 are part of the description.

The claims are to follow.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Qiagen GmbH
(B) STREET: Max-Volmer-Strasse 4
(C) CITY: Hilden
(E) COUNTRY: DE
(F) POSTAL CODE (ZIP): 40724

(ii) TITLE OF INVENTION: New method for the isolation of ccc plasmid DNA

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4618 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7612 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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AGAGCGGCCG CCACGGCGAT ATCGGATCCA TATGACGTCG ACGCGTCTGC AG 7612

8

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for the production of biomass for the isolation of ccc-plasmid DNA comprising
   (a) culturing a bacterial transformant in a bio-reactor containing an antibiotic-free batch-medium comprising
      (aa) a carbon source;
      (ab) an inorganic salt mixture;
      (ac) a nitrogen source;
      under batch-culturing conditions;
   (b) feeding under feed-back conditions to said culture of (a) at the end of the batch phase, after rising of the dissolved oxygen above a threshold-set point, a portion of a feed-medium comprising
      (ba) a carbon source; and
      (bb) a magnesium salt; and
   (c) allowing the bacterial transformant to metabolize said feed-medium.

2. The method according to claim 1, wherein step (b) comprises repeated feeding cycles after each rising of the dissolved oxygen above a threshold-set point.

3. The method according to claim 1 and 2, wherein the bacterial transformant is an *E. coli* cell.

4. The method according to claim 1 to 3, wherein glycerol is used as a carbon source.

5. The method according to anyone of claims 1 to 4, wherein NH_3 is used as a nitrogen source.

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6. The method according to anyone of claims 1 to 5, wherein the carbon source in the batch-medium is in a concentration of ≤ 100 g/l.

7. The method according to anyone of claims 1 to 6, wherein the carbon source in the feed-medium is in a concentration of ≤ 1000 g/l.

8. The method according to anyone of claims 1 to 7, wherein the nitrogen source is in a concentration of ≤ 30%.

9. The method according to anyone of claims 1 to 8, wherein the inorganic salt mixture comprises Na_2HPO_4 ≤ 6 g/l, KH_2PO_4 ≤ 3 g/l, NaCl ≤ 0,5 g/l and citric acid•H_2O ≤ 1,5 g/l.

10. The method according to claim 9, wherein said inorganic salt mixture also comprises a magnesium salt.

11. The method according to claim 10, wherein said magnesium salt is $MgSO_4$, in a concentration of 0.3 g/l.

12. The method according to anyone of claims 1 to 11, wherein in step (b), the magnesium salt concentration is in a range of 5 to 100 mM.

13. The method according to claim 12, wherein in step (b) magnesium salt concentration is 80 mM.

14. The method according to anyone of claims 1 to 13, wherein the magnesium salt is $MgSO_4$.

15. The method according to anyone of claims 1 to 14, wherein a solution of trace elements is added in step (a) and/or (b).

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16. The method according to anyone of claims 1 to 15, wherein the solution of trace elements comprises: $FeCl_3$ •6 H_2O, $ZnSO_4$ •7 H_2O, $MnSO_4$ •, $CoSO_4$ • 7 H_2O, $CuCl_2$, H_3BO_3, Na_2MoO4 • 2 H_2O and citric acid.

17. The method according to anyone of claims 1 to 16, wherein the batch-medium comprises an amino acid source.

18. The method according to anyone of claims 1 to 17, wherein the feed-medium comprises an amino acid source.

19. The method according to anyone of claims 1 to 18, wherein the culturing of the bacterial transformant is carried out at a temperature range of 30°C to 42°C.

20. The method according to claims 1 to 19, wherein the temperature range is about 35°C to 38°C.

21. The method according to anyone of claims 1 to 20, wherein the batch-medium comprises a bacterial host strain specific supplement.

22. The method according to anyone of claims 1 to 21, wherein the host cells, after step (c), are harvested from said cultures.

23. The method according to claim 22, wherein the host cells are, after step (c), subjected to a washing step before or after harvesting.

24. The method according to anyone of claims 1 to 23, wherein a further step, after step (c), comprises the freezing or freeze-drying of the host cells.

25. The method according to anyone of claims 1 to 24, wherein a further step, after step (c) or after the steps specified in claims 22 to 24, comprises the isolation of ccc DNA.

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26. The method according to anyone of claims 1 to 25, further comprising the step of (a') pre-culturing the bacterial transformant in an antibiotic-free medium.

27. The method according to claim 26, wherein the bacterial transformant is in exponential growth phase after the end of said pre-culturing.

28. The method according to any one of claims 1 to 27 wherein the batch-medium, the feed-medium and/or the medium used for pre-culturing is a synthetic or a semisynthetic medium.

29. A method according to any one of claims 1 to 28 substantially as hereinbefore described with reference to the drawings and/or examples.

DATED this 5th day of August 2002

Qiagen GmbH

by DAVIES COLLISON CAVE
Patent Attorneys for the Applicants

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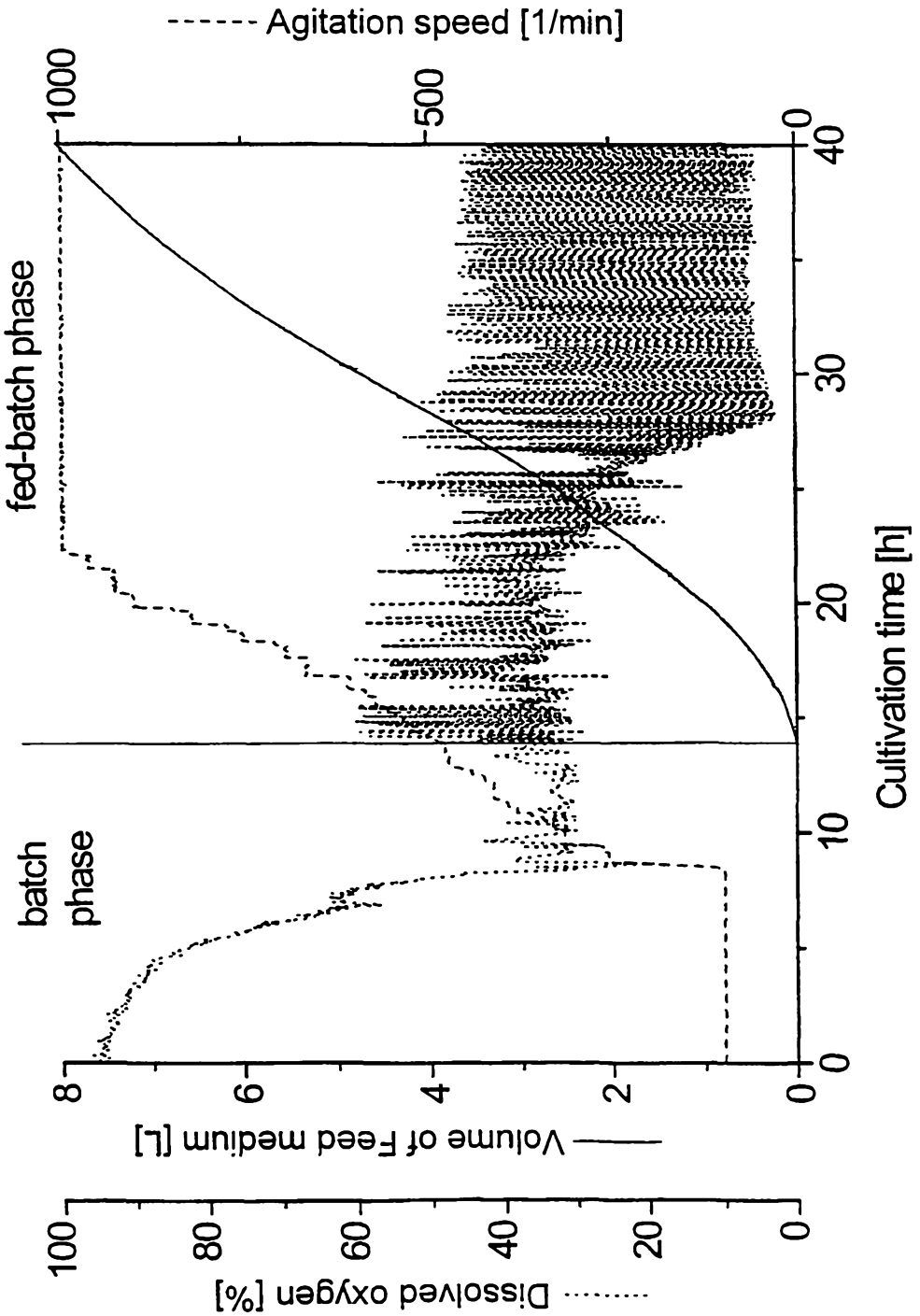
Fig. 1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fed-batch phase
batch phase
Cultivation time [h]
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4
6
8
Dissolved oxygen [%]
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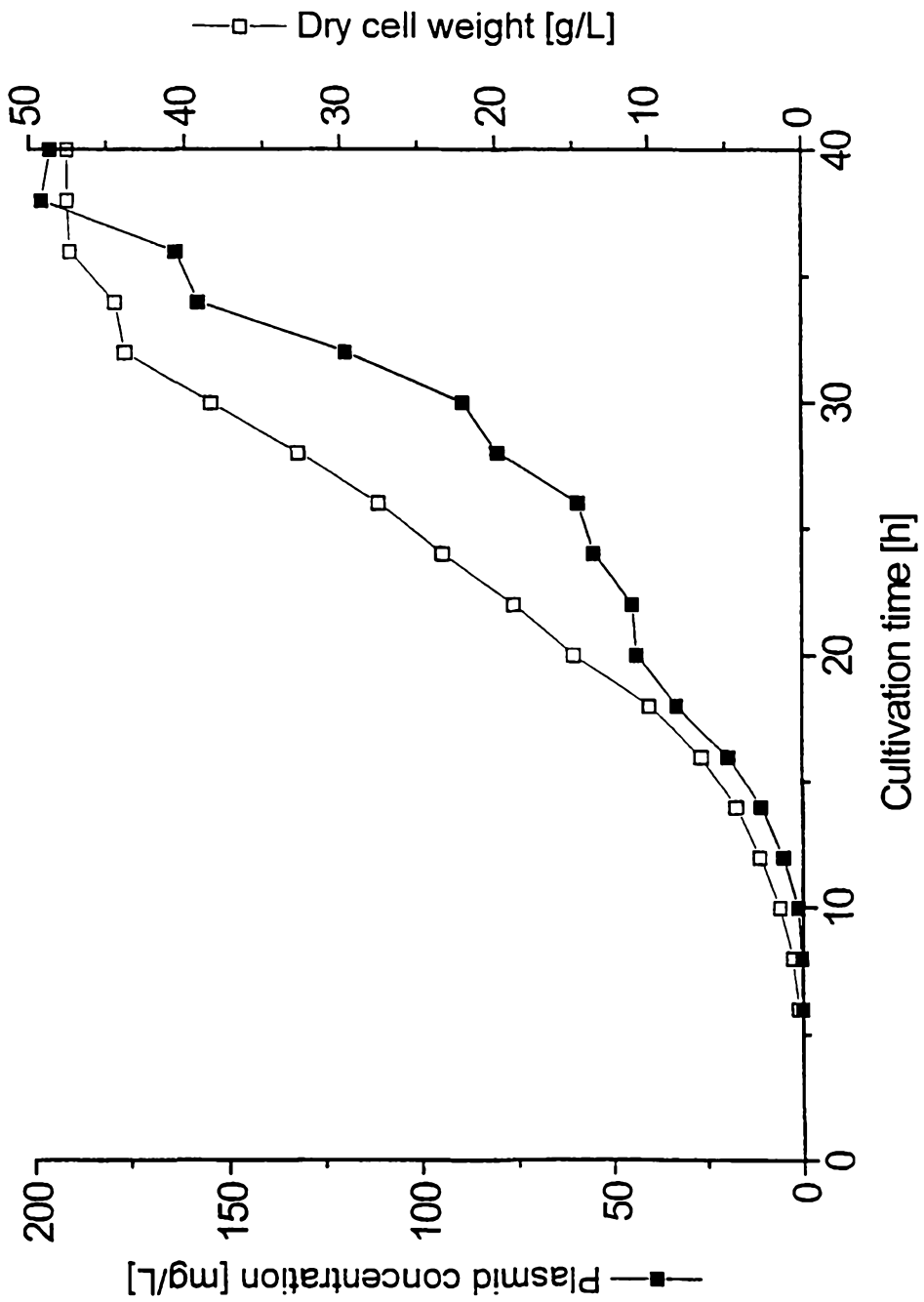
20
40
60
80
100

Fig. 2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30
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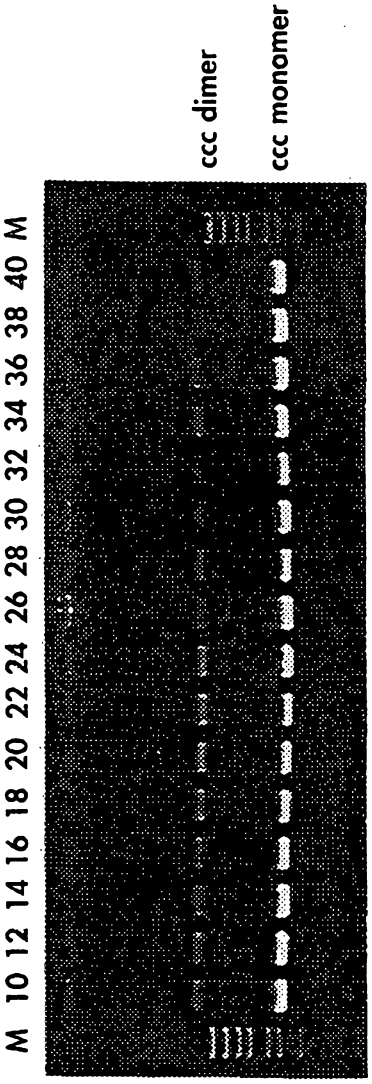
Fig. 3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ccc monomer
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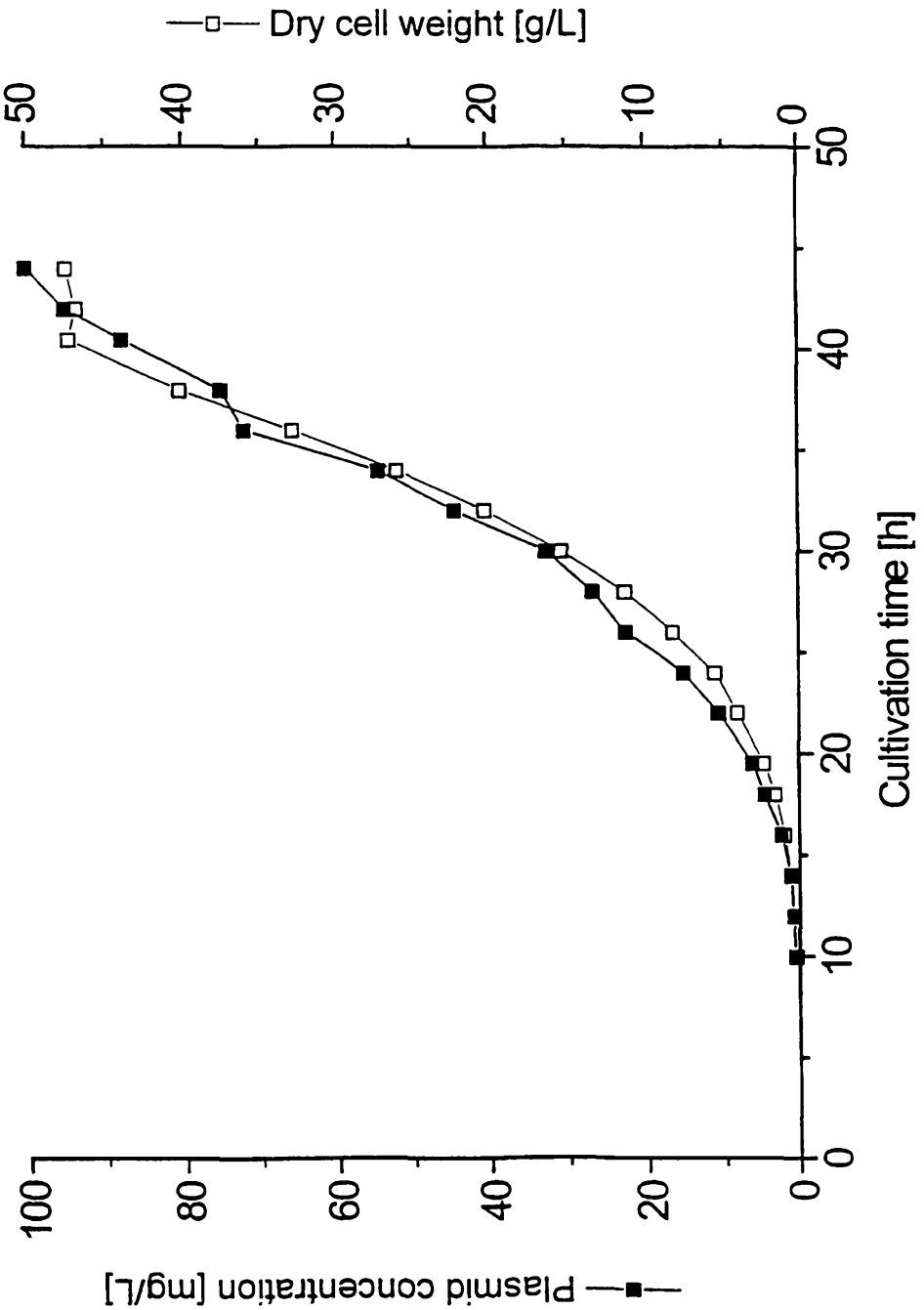
Fig. 4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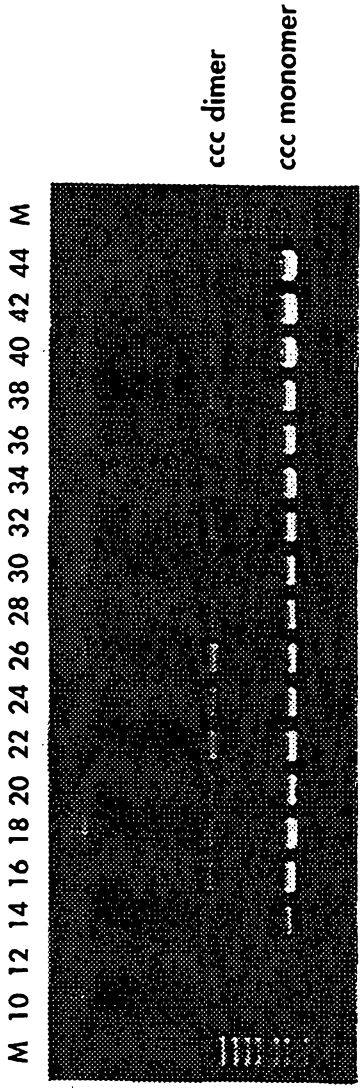
20
0
Plasmid concentration [mg/L]

Fig. 5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ccc monomer
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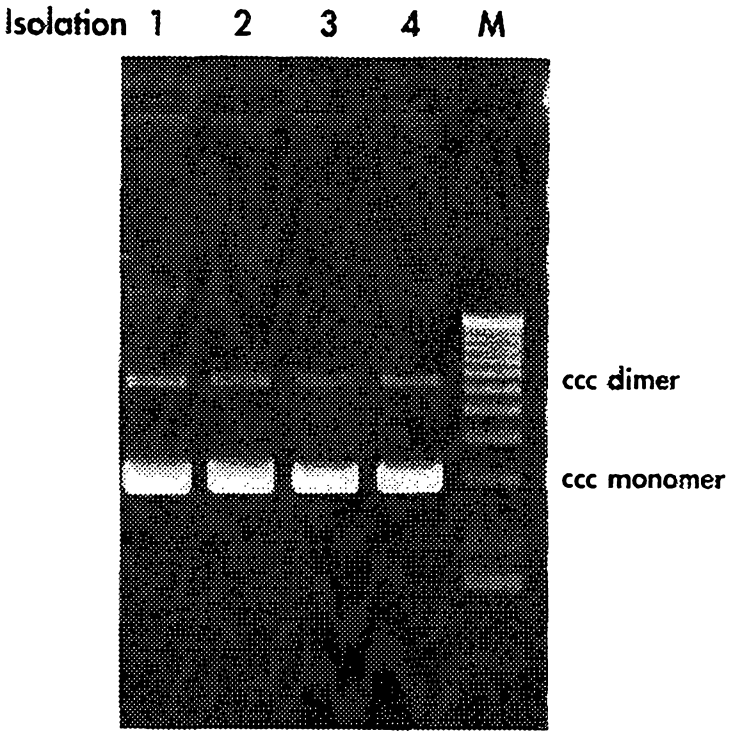

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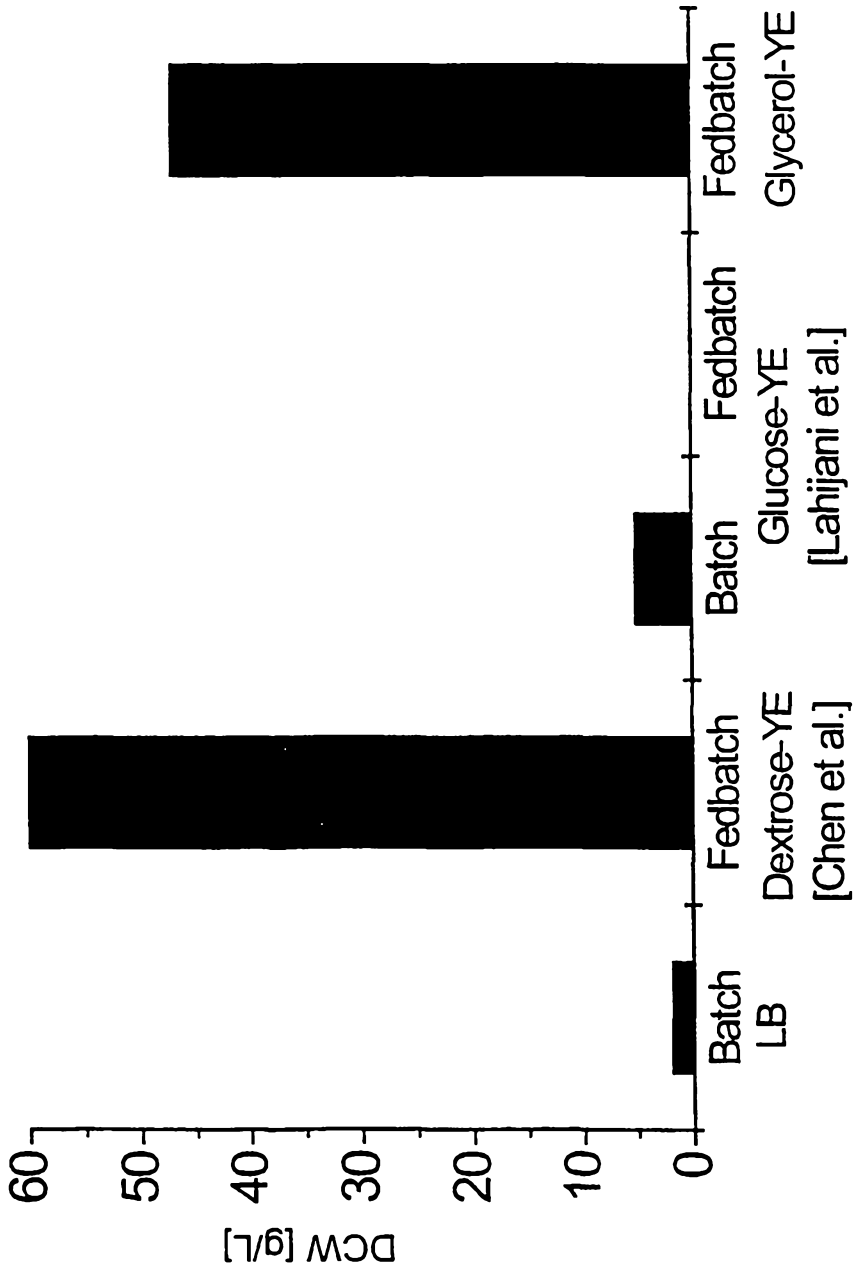
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50
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LB
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[Chen et al.]
Batch
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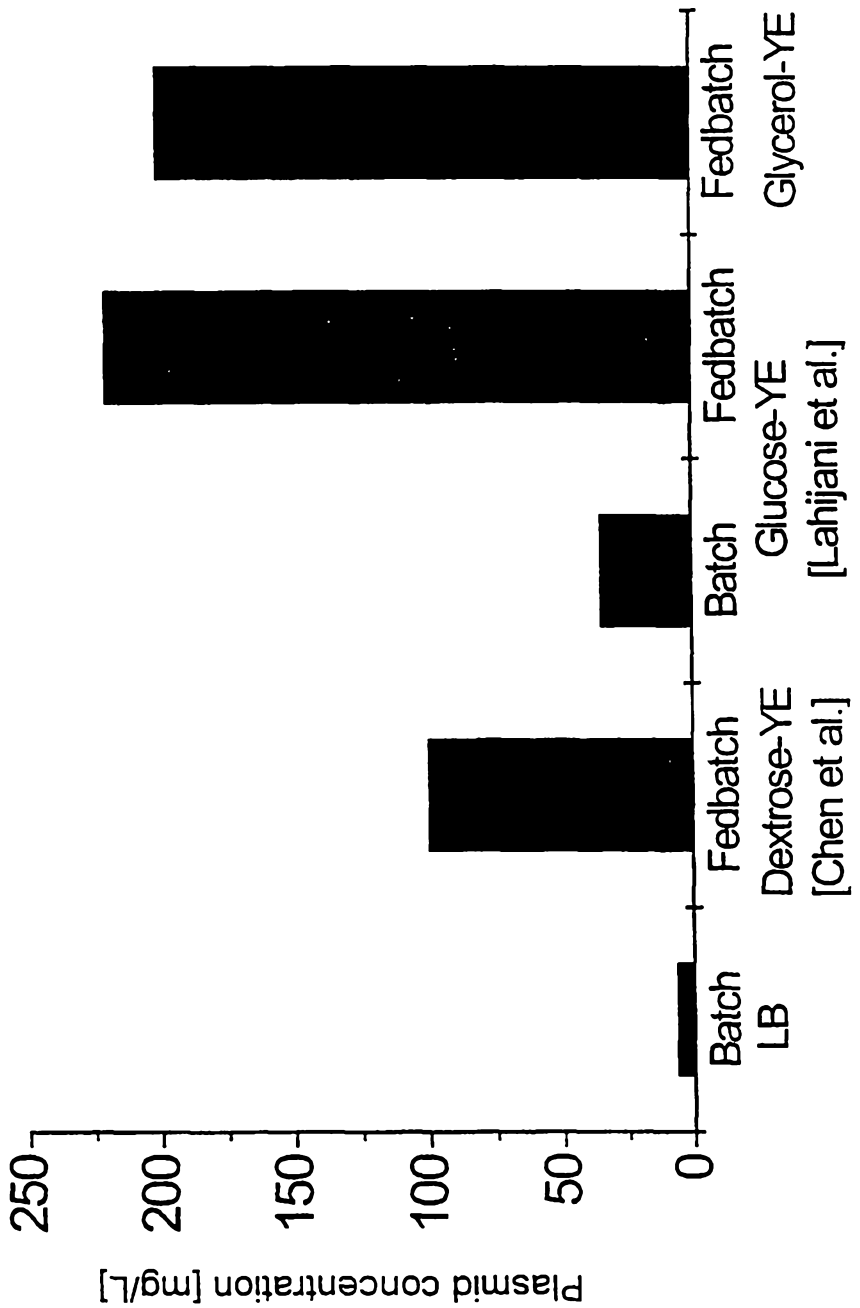
[Lahijani et al.]
Fedbatch
Fedbatch
Glycerol-YE

Fig. 8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100
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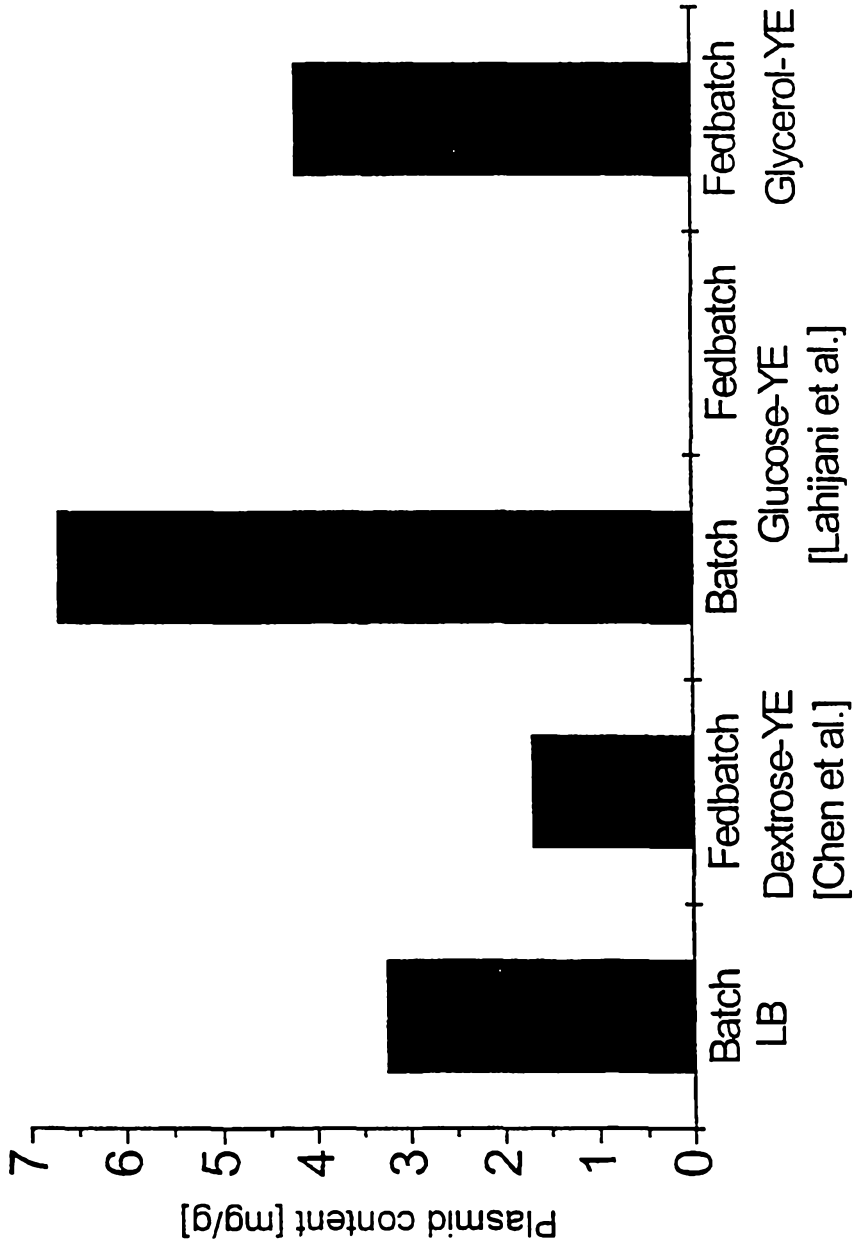
[Chen et al.]
Batch
Glucose-YE
Fedbatch
[Lahijani et al.]
Fedbatch
Glycerol-YE

Fig. 9


Plasmid content [mg/g]
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3
4
5
6
7
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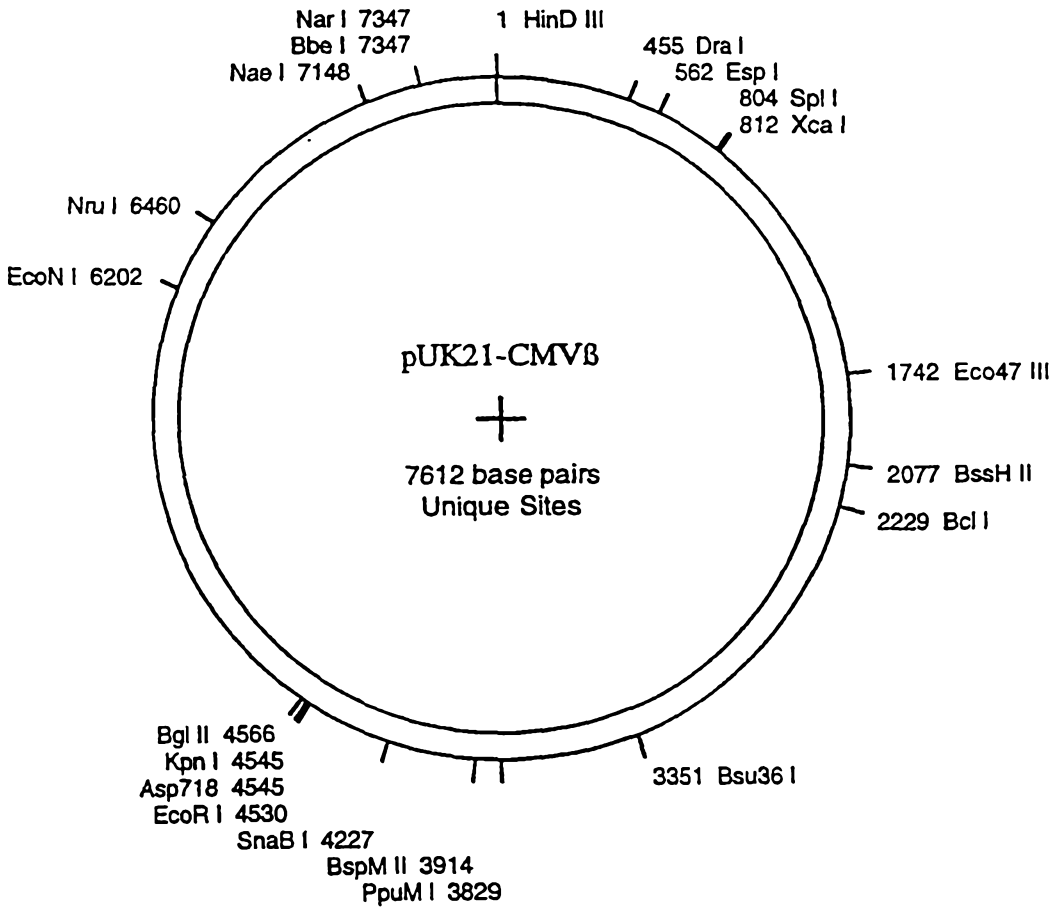
LB
Fedbatch
Dextrose-YE
[Chen et al.]
Batch
Glucose-YE
[Lahijani et al.]
Fedbatch
Fedbatch
Glycerol-YE

Fig. 10



The 4,5 kbp EcoRI-HindIII-restriction fragment of pCMVß (Clontech Laboratories; , Palo Alto, California, USA), containing the ß-galactosidase gene under the control of the CMV-promoter, was cloned into the corresponding restriction sites of pUK21 (Vieira, J., and Messing, J., 1991, Gene 100:189-194).

pUK21-CMVß

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Fig. 11

Fig. 11 cont.

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Fig. 11 cont.

Fig. 11 cont.

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Fig. 11 cont.

Fig. 11 cont.

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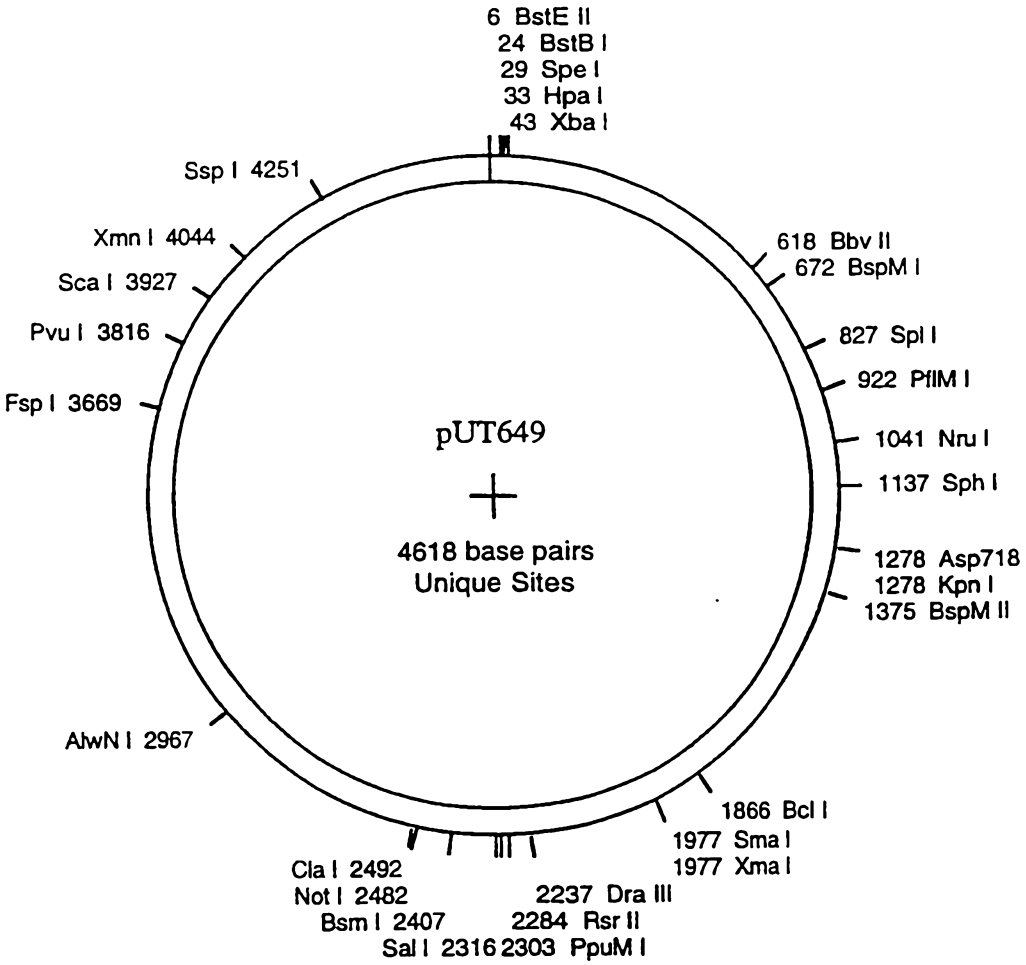
7441 TGCAAGGCGA TTAAGTTGGG TAACGCCAGG  GTTTTCCCAG TCACGACGTT GTAAAACGAC 7500
7501 GGCCAGTGAA TTGTAATACG ACTCACTATA  GGGCGAATTG GGGATCGATC CACTAGTTCT 7560
7561 AGAGCGGCCG CCACGGCGAT ATCGGATCCA  TATGACGTCG ACGCGTCTGC AG         7612
          |   10      |   20      |   30      |   40      |   50      |   60
```

Fig. 12


6 BstE II
24 BstB I
29 Spe I
33 Hpa I
43 Xba I
618 Bbv II
672 BspM I
827 Spl I
922 PflM I
1041 Nru I
1137 Sph I
1278 Asp718
1278 Kpn I
1375 BspM II
1866 Bcl I
1977 Sma I
1977 Xma I
2237 Dra III
2284 Rsr II
2303 PpuM I
Sal I 2316
Bsm I 2407
Not I 2482
Cla I 2492
AlwN I 2967
Fsp I 3669
Pvu I 3816
Sca I 3927
Xmn I 4044
Ssp I 4251
pUT649
4618 base pairs
Unique Sites

pUT649

```
        |    10      |    20      |    30      |    40      |    50      |    60
   1 CATATGGTGA CCGGATCCAC GCGTTCGAAC TAGTTAACTA GATCTAGAGT CCGTTACATA 60
  61 ACTTACGGTA AATGGCCCGC CTGGCTGACC GCCCAACGAC CCCCGCCCAT TGACGTCAAT 120
 121 AATGACGTAT GTTCCCATAG TAACGCCAAT AGGGACTTTC CATTGACGTC AATGGGTGGA 180
 181 GTATTTACGG TAAACTGCCC ACTTGGCAGT ACATCAAGTG TATCATATGC CAAGTACGCC 240
 241 CCCTATTGAC GTCAATGACG GTAAATGGCC CGCCTGGCAT TATGCCCAGT ACATGACCTT 300
 301 ATGGGACTTT CCTACTTGGC AGTACATCTA CGTATTAGTC ATCGCTATTA CCATGGTGAT 360
 361 GCGGTTTTGG CAGTACATCA ATGGGCGTGG ATAGCGGTTT GACTCACGGG GATTTCCAAG 420
 421 TCTCCACCCC ATTGACGTCA ATGGGAGTTT GTTTTGGCAC CAAAATCAAC GGGACTTTCC 480
 481 AAAATGTCGT AACAACTCCG CCCCATTGAC GCAAATGGGC GGTAGGCGTG TACGGTGGGA 540
 541 GGTCTATATA AGCAGAGCTC GTTTAGTGAA CCGTCAGATC GCCTGGAGAC GCCATCCACG 600
 601 CTGTTTTGAC CTCCATAGAA GACACCGGGA CCGATCCAGC CTCCGCGGCC GGGAACGGTG 660
 661 CATTGGAACG GACCTGCAGC ACGTGTTGAC AATTAATCAT CGGCATAGTA TATCGGCATA 720
 721 GTATAATACG ACTCACTATA GGAGGGCCAC CATGGCCTCG TACCCCGGCC ATCAACACGC 780
 781 GTCTGCGTTC GACCAGGCTG CGCGTTCTCG CGGCCATAGC AACCGACGTA CGGCGTTGCG 840
 841 CCCTCGCCGG CAGCAAGAAG CCACGGAAGT CCGCCCGGAG CAGAAAATGC CCACGCTACT 900
 901 GCGGGTTTAT ATAGACGGTC CCCACGGGAT GGGGAAAACC ACCACCACGC AACTGCTGGT 960
 961 GGCCCTGGGT TCGCGCGACG ATATCGTCTA CGTACCCGAG CCGATGACTT ACTGGCGGGT 1020
1021 GCTGGGGGCT TCCGAGACAA TCGCGAACAT CTACACCACA CAACACCGCC TCGACCAGGG 1080
1081 TGAGATATCG GCCGGGGACG CGGCGGTGGT AATGACAAGC GCCCAGATAA CAATGGGCAT 1140
```

Fig. 13

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1141 GCCTTATGCC GTGACCGACG CCGTTCTGGC TCCTCATATC GGGGGGGAGG CTGGGAGCTC 1200
1201 ACATGCCCCG CCCCCGGCCC TCACCCTCAT CTTCGACCGC CATCCCATCG CCGCCCTCCT 1260
1261 GTGCTACCCG GCCGCGCGGT ACCTTATGGG CAGCATGACC CCCCAGGCCG TGCTGGCGTT 1320
1321 CGTGGCCCTC ATCCCGCCGA CCTTGCCCGG CACCAACATC GTGCTTGGGG CCCTTCCGGA 1380
1381 GGACAGACAC ATCGACCGCC TGGCCAAACG CCAGCGCCCC GGCGAGCGGC TGGACCTGGC 1440
1441 TATGCTGGCT GCGATTCGCC GCGTTTACGG GCTACTTGCC AATACGGTGC GGTATCTGCA 1500
1501 GTGCGGCGGG TCGTGGCGGG AGGACTGGGG ACAGCTTTCG GGGACGGCCG TGCCGCCCCA 1560
1561 GGGTGCCGAG CCCCAGAGCA ACGCGGGCCC ACGACCCCAT ATCGGGGACA CGTTATTTAC 1620
1621 CCTGTTTCGG GCCCCCGAGT TGCTGGCCCC CAACGGCGAC CTGTATAACG TGTTTGCCTG 1680
1681 GGCCTTGGAC GTCTTGGCCA AACGCCTCCG TTCCATGCAC GTCTTTATCC TGGATTACGA 1740
1741 CCAATCGCCC GCCGGCTGCC GGGACGCCCT GCTGCAACTT ACCTCCGGGA TGGTCCAGAC 1800
1801 CCACGTCACC ACCCCCGGCT CCATACCGAC GATATGCGAC CTGGCGCGCA CGTTTGCCCG 1860
1861 TGAGATGATC AGCGGAGCTA ATGGCGTCAT GGCCAAGTTG ACCAGTGCCG TTCCGGTGCT 1920
1921 CACCGCGCGC GACGTCGCCG GAGCGGTCGA GTTCTGGACC GACCGGCTCG GGTTCTCCCG 1980
1981 GGACTTCGTG GAGGACGACT TCGCCGGTGT GGTCCGGGAC GACGTGACCC TGTTCATCAG 2040
2041 CGCGGTCCAG GACCAGGTGG TGCCGGACAA CACCCTGGCC TGGGTGTGGG TGCGCGGCCT 2100
2101 GGACGAGCTG TACGCCGAGT GGTCGGAGGT CGTGTCCACG AACTTCCGGG ACGCCTCCGG 2160
2161 GCCGGCCATG ACCGAGATCG GCGAGCAGCC GTGGGGGCGG GAGTTCGCCC TGCGCGACCC 2220
2221 GGCCGGCAAC TGCGTGCACT TCGTGGCCGA GGAGCAGGAC TGACCGACGC CGACCAACAC 2280
2281 CGCCGGTCCG ACGGCGGCCC ACGGGTCCCA GGGGGGTCGA CCTCGAAACT TGTTTATTGC 2340
2341 AGCTTATAAT GGTTACAAAT AAAGCAATAG CATCACAAAT TTCACAAATA AAGCATTTTT 2400
2401 TTCACTGCAT TCTAGTTGTG GTTTGTCCAA ACTCATCAAT GTATCTTATC ATGTCTGGAT 2460
2461 CCCTCGGAGA TCTGGGCCCA TGCGGCCGCG GATCGATGCT CACTCAAAGG CGGTAATACG 2520
2521 GTTATCCACA GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA 2580
2581 GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC GCCCCCCTGA 2640
2641 CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG GACTATAAAG 2700
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Fig. 13 cont.

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2701 ATACCAGGCG TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT 2760
2761 TACCGGATAC CTGTCCGCCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC AATGCTCACG 2820
2821 CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC 2880
2881 CCCCGTTCAG CCCGACCGCT GCGCCTTATC CGGTAACTAT CGTCTTGAGT CCAACCCGGT 2940
2941 AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA GAGCGAGGTA 3000
3001 TGTAGGCGGT GCTACAGAGT TCTTGAAGTG GTGGCCTAAC TACGGCTACA CTAGAAGGAC 3060
3061 AGTATTTGGT ATCTGCGCTC TGCTGAAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC 3120
3121 TTGATCCGGC AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT 3180
3181 TACGCGCAGA AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC 3240
3241 TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA AAAGGATCTT 3300
3301 CACCTAGATC CTTTTAAATT AAAAATGAAG TTTTAAATCA ATCTAAAGTA TATATGAGTA 3360
3361 AACTTGGTCT GACAGTTACC AATGCTTAAT CAGTGAGGCA CCTATCTCAG CGATCTGTCT 3420
3421 ATTTCGTTCA TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACTACGA TACGGGAGGG 3480
3481 CTTACCATCT GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC CGGCTCCAGA 3540
3541 TTTATCAGCA ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC CTGCAACTTT 3600
3601 ATCCGCCTCC ATCCAGTCTA TTAATTGTTG CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT 3660
3661 TAATAGTTTG CGCAACGTTG TTGCCATTGC TACAGGCATC GTGGTGTCAC GCTCGTCGTT 3720
3721 TGGTATGGCT TCATTCAGCT CCGGTTCCCA ACGATCAAGG CGAGTTACAT GATCCCCCAT 3780
3781 GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA GTAAGTTGGC 3840
3841 CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG TCATGCCATC 3900
3901 CGTAAGATGC TTTTCTGTGA CTGGTGAGTA CTCAACCAAG TCATTCTGAG AATAGTGTAT 3960
3961 GCGGCGACCG AGTTGCTCTT GCCCGGCGTC AATACGGGAT AATACCGCGC CACATAGCAG 4020
4021 AACTTTAAAA GTGCTCATCA TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT 4080
4081 ACCGCTGTTG AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT CTTCAGCATC 4140
4141 TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG CCGCAAAAAA 4200
4201 GGGAATAAGG GCGACACGGA AATGTTGAAT ACTCATACTC TTCCTTTTTC AATATTATTG 4260
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Fig. 13 cont.

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4261 AAGCATTTAT CAGGGTTATT GTCTCATGAG CGGATACATA TTTGAATGTA TTTAGAAAAA 4320
4321 TAAACAAATA GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTGACG TCTAAGAAAC 4380
4381 CATTATTATC ATGACATTAA CCTATAAAAA TAGGCGTATC ACGAGGCCCT TTCGTCTCGC 4440
4441 GCGTTTCGGT GATGACGGTG AAAACCTCTG ACACATGCAG CTCCCGGAGA CGGTCACAGC 4500
4501 TTGTCTGTAA GCGGATGCCG GGAGCAGACA AGCCCGTCAG GGCGCGTCAG CGGGTGTTGG 4560
4561 CGGGTGTCGG GGCTGGCTTA ACTATGCGGC ATCAGAGCAG ATTGTACTGA GAGTGCAC   4618
         |   10      |   20      |   30      |   40      |   50      |   60
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Fig. 13 cont.