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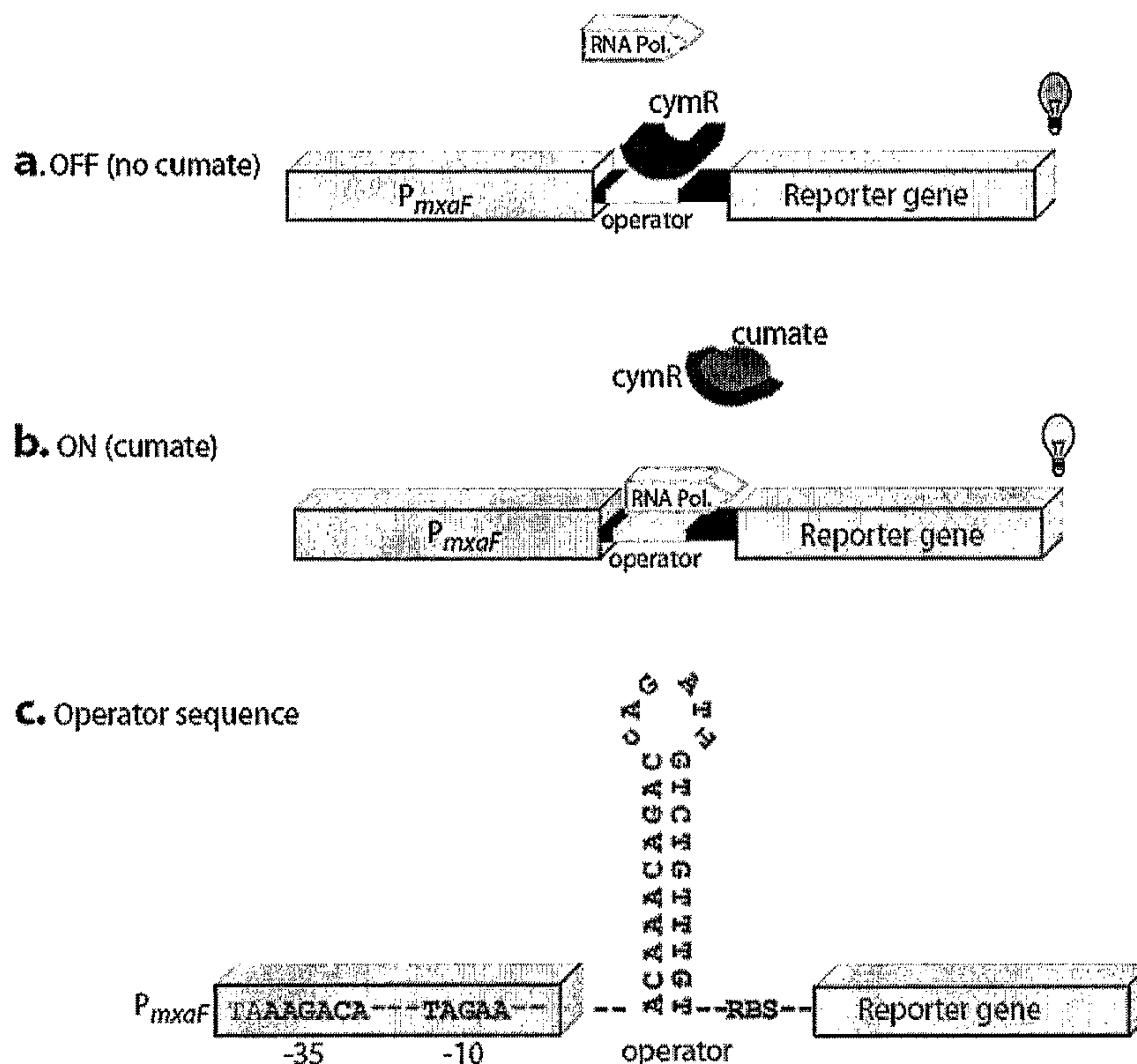
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(54) Title: REGULATION OF HETEROLOGOUS RECOMBINANT PROTEIN EXPRESSION IN METHYLOTROPHIC AND METHANOTROPHIC BACTERIA



(57) **Abrégé/Abstract:**

Methylotrophic or methanotrophic bacteria such as *Methylobacterium* are transformed with a gene of interest, and expression of the gene is regulated by means of a cumate repressor protein and an operator sequence which is operatively linked to the gene of

(57) **Abrégé(suite)/Abstract(continued):**

interest, and the addition of an external agent. Specifically, the cymR repressor and cmt operator from *Pseudomonas putida* may serve to regulate gene expression in methylotrophic or methanotrophic bacteria with the addition of cumate.

(57) Abstract: Methylotrophic or methanotrophic bacteria such as *Methylobacterium* are transformed with a gene of interest, and expression of the gene is regulated by means of a cumate repressor protein and an operator sequence which is operatively linked to the gene of interest, and the addition of an external agent. Specifically, the *cymR* repressor and *cmt* operator from *Pseudomonas putida* may serve to regulate gene expression in methylotrophic or methanotrophic bacteria with the addition of cumate.

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**REGULATION OF HETEROLOGOUS RECOMBINANT PROTEIN
EXPRESSION IN METHYLOTROPHIC AND METHANOTROPHIC
BACTERIA**

5 FIELD OF THE INVENTION

The invention relates to the regulation of gene expression in transformant methylotrophic or methanotrophic cells by means of a repressor-operator system.

BACKGROUND OF THE INVENTION

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Methylotrophic bacteria are ubiquitous, inhabiting different aquatic and terrestrial habitats including the phyllosphere. In addition to their important role in the cycling of single carbon compounds in the environment, methanol-utilizing methylotrophs have been shown to enter into plant-growth-promoting phytosymbiotic relationships (1, 2). Amongst the methylotrophs, several *Methylobacterium* species have been well described in the literature. One of the best characterized strains within the *Methylobacterium* genus, both genetically and physiologically, is the methanol utilizing *Methylobacterium extorquens* AM1 (3). Another well characterized *M. extorquens* strain, ATCC 55366, was isolated for its capacity to produce high quantities of poly- β -hydroxybutyric acid. A fed-batch fermentation system was developed specifically for this strain to further enhance its growth potential and PHB productivity. See also U.S. Patent no. 5,302,525, which issued Nov. 23, 1992.

Methylobacterium extorquens has received special attention as a potential source of industrially pertinent natural and recombinant proteins (4, 5). The industrial potential of *M. extorquens* as a producer of recombinant proteins is due to: (i) the simplicity of its growth requirements and a comparably inexpensive substrate (methanol); (ii) the development and optimization of protocols for high cell density cultivation; (iii) the sequencing and annotation of the *M. extorquens* AM1 genome; and (iv) the development of genetic tools specifically for *M. extorquens* comprising novel cloning and expression vectors, efficient transposon mutagenesis, integrative expression vectors and protocols, and simple and efficient electroporation protocols (6-8). The potential of *M. extorquens* and other pink pigmented facultative methylotrophs (PPFMs) as “cell factories” is

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further enhanced by their inherent capabilities to produce natural products of great import including pyrroloquinoline quinone (9, 10), vitamin B12 (11), poly- β -hydroxybutyric acid (12, 13), caratenoids (14), and phytohormones (15, 16). By applying the molecular tools and high cell-density fermentation technologies mentioned
5 above, it has been possible to overexpress a variety of recombinant proteins in *M. extorquens* strains ATCC 55366 and AM1. Representative proteins overexpressed in these two *M. extorquens* strains include the green fluorescent protein, esterase from *Lactobacillus casei*, enterocin P from *Enterococcus faecium*, and haloalkane dehalogenase from *Xanthobacter autotrophicus* (4, 17, 18, 19). Several of these
10 activities are the subject of our pending U.S. Patent Publication No. 2006 0234336, filed November 29, 2002. Miguez et al.

However, as it concerns *Methylobacterium extorquens* strains AM1 and ATCC 55366, practical and tight inducible expression of recombinant genes has not been attained (20).
15 Inducible heterologous promoters have been tested in *M. extorquens* ATCC 55366, in particular P_{lac} and λP_L and P_R however expression is both leaky and weak. *M. extorquens* possesses native methanol inducible promoters, notably promoters which are located upstream of genes that encode for the methanol dehydrogenase and other proteins required for its activity, and for enzymes required for
20 the synthesis of the methanol dehydrogenase prosthetic group, the pyrroloquinoline quinone. Of these, promoter P_{mxoF} has been thoroughly scrutinized both biochemically and in expression studies. Although it is a very strong promoter (4, 17, 20), in practical terms it is essentially constitutive. Expression of the green fluorescent gene (*gfp*) under the control of P_{mxoF} occurred even when the culture was grown repeatedly on succinate.

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SUMMARY OF THE INVENTION

A first object of the invention is to provide a method for regulating the production of recombinant proteins in methylotrophic or methanotrophic bacteria such as
30 *Methylobacterium*. This may be done by means of a repressor-operator system that is operatively linked to a gene of interest.

A further object of the invention is to provide a method for regulating heterologous gene expression in *Methylobacterium extorquens*.

5 A first aspect of the invention provides for a polynucleotide construct for use in association with a methylotrophic or methanotrophic host cell, the host cell comprising a nucleotide that encodes a cumate repressor protein exhibiting repressor activity which is regulatable by the addition of an external agent, and the polynucleotide construct comprising a promoter fused to an operator sequence which is operatively linked to at least one gene of interest, wherein the operator sequence is
10 in operative association with the cumate repressor protein and wherein addition of the external agent upregulates expression of the at least one gene of interest.

A second aspect of the invention provides for a methylotrophic or methanotrophic host cell comprising a nucleotide that encodes a cumate repressor protein exhibiting
15 repressor activity which is regulatable by the addition of an external agent, and further comprising a polynucleotide construct which comprises a promoter fused to an operator sequence which is operatively linked to at least one gene of interest, wherein the operator sequence is in operative association with the cumate repressor protein and wherein addition of the external agent upregulates expression of the at least one
20 gene of interest.

A third aspect of the invention provides for a gene expression system comprising a methylotrophic or methanotrophic host cell comprising a nucleotide that encodes a p-cumate repressor protein exhibiting repressor activity which is regulatable by the
25 addition of an external agent, and further comprising a polynucleotide construct which comprises a promoter fused to an operator sequence which is operatively linked to at least one gene of interest, wherein the operator sequence is in operative association with the cumate repressor protein and wherein addition of the external agent upregulates expression of the at least one gene of interest.

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A further aspect of the invention provides for a method for selectively controlling the expression of at least one gene of interest comprising the steps of a) obtaining a methylotrophic or methanotrophic host cell comprising a nucleotide that encodes a cumate repressor protein exhibiting repressor activity which is regulatable by the

addition of an external agent, and further comprising a polynucleotide construct which comprises a promoter fused to an operator sequence which is operatively linked to at least one gene of interest, wherein the operator sequence is in operative association with the cumate repressor protein and wherein expression of the at least one gene of interest is repressed due to the repressor activity of the repressor protein; and b) exposing the host cell to an external agent which inhibits the repressor activity and allows for expression of the at least one gene of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1.** A schematic diagram of the mechanism of action of the cumate-switchable expression system. (a) In the absence of the inducer, cumate, the repressor protein (cymR) is bound to the operator site upstream of the reporter gene or gene of interest, and inhibits transcription. (b) The presence of the cumate is necessary for transcription of gene of interest. The addition of cumate rapidly alters the binding of the operator to the repressor cymR, thereby facilitating the formation of the cymR-cumate complex. The detachment of the cymR from the operator activates transcription of the downstream reporter gene. The cymR-cumate complex is unable to bind to operator site. (c) Nucleotide sequence of the operator, which represents the putative binding site for the helix-turn-helix transcription factor.
- Figure 2.** Analysis of intracellular polypeptides from recombinant strains of *M. extorquens*::cymR expressing repressor protein. (a) Western blot analysis of repressor protein expressed by native *M. extorquens*, single copy of cymR integrant and double copy cymR integrant, respectively. Arrows indicate the position and the molecular size (Kda) of the repressor protein. (b) Colony PCR profiles. The physical presence of the integrated cymR gene in the chromosome of *M. extorquens* was tested in 8 randomly selected clones (cymR2) by colony PCR utilizing the primers described in the experimental protocols. The position and the size of the expected PCR products are marked. P_{Tn7LR}, P_{dhaTF}, P_{Tn7RF} and P_{glmSR} represent the primers used to generate PCR products. (c) The presence of the repressor protein in 8 randomly selected clones (see b) was confirmed by Western blot analysis.

Figure 3. Comparison of growth profiles between native *M. extorquens* and modified *M. extorquens* expressing cymR. Chromosomal integration and expression of cymR using the Tn7 system does not appear to negatively affect the growth and metabolism of *M. extorquens*. The growth profiles of the native or recombinant strains are indistinguishable, indicating that the repressor protein is not toxic to the host, and the modified recombinant host strain can be used as a new host strain for cumate-inducible expression system.

Figure 4. The maps of recombinant plasmids containing the required regulatory elements. Abbreviations: *traJ'*, mutated conjugal transfer gene that is missing the final 85 of 123 amino acids; *oriT*, origin of transfer; *oriV*, vegetative origin; *trfA*, replication initiator gene; Km^r, kanamycin resistance gene; P_{mxoF}⁺op, methanol dehydrogenase combined with operator sequence from *P. putida* F1; *gfp*, green fluorescent protein encoding gene; *estI*, esterase encoding gene; and *bgl*, β -galactosidase encoding gene.

Figure 5. Determination of cumate concentration required for optimal induction of recombinant *gfp* gene expression. The results indicate that the system is very finely controlled. The addition of 1 μ g cumate/ml results in 47% induction of gene expression. The addition of 15-30 μ g cumate/ml results in maximum gene expression.

Figure 6. Identification of the optimal time of induction. Maximum gene expression was obtained when the culture was induced with 15 μ g cumate/ml at an OD₆₀₀ of 0.8-1.0. Induction at OD₆₀₀ of 0.4, 1.5, and 2.0 resulted in reduced expression of the target gene.

Figure 7. Induction and production of recombinant proteins by the cumate-regulated expression system. The inducible expression system was validated with three different heterologous genes; Green Fluorescent Protein (A), β -galactosidase (B) and esterase (C). Without cumate induction, target proteins were not detected, as analyzed by UV for GFP, chromogenic substrate (x-gal) for β -galactosidase, and SDS-PAGE for esterase. Lower lane shows the recombinant protein production profiles from each construct. Arrow indicates the point of induction with cumate (15 μ g/ml).

DETAILED DESCRIPTION OF THE INVENTION

The invention disclosed relates to the development of a methylotrophic or
5 methanotrophic host cell recombinant protein expression system. More specifically,
the methylotrophic or methanotrophic host cell may be of the genus
Methylobacterium, and even more specifically may be of the species
Methylobacterium extorquens. It is desirable to be able to control the production of a
recombinant protein, as constitutive production of the protein may not be desirable.
10 Such control may be achieved using a repressor-operator regulation system such as
the regulatory elements of the *Pseudomonas putida* F1, namely the *cym* and *cmt*
operons, enabling inducible/regulated gene expression.

First, the gene encoding for a repressor protein, such as *cymR*, is integrated into the
chromosome of the methylotrophic or methanotrophic host cell downstream of a
15 strong native promoter. Second, an expression vector is constructed by inserting a
strong native constitutive promoter, such as $P_{mx\alpha F}$ or other suitable promoter such as λ ,
and the repressor protein binding sites or operator region downstream of the
transcription start site for a gene of interest, such as a reporter gene. This vector is
then transferred by electroporation to the host cell containing the chromosomally
20 integrated repressor protein gene. Addition of an external agent or inducer to the
culture prevents the binding of the repressor to the operator region thereby alleviating
transcription repression and resulting in the tight *inducible* expression of the reporter
gene. While the experimental data discussed herein relates to *Methylobacterium*
extorquens host cells, it is expected that the method should work in other
25 *Methylobacterium* strains as well as other methylotrophic bacteria, due to the high
degree of genetic similarity between the strains. Further, the use of the strong native
promoter $P_{mx\alpha F}$ produced good results, and it is known that the $P_{mx\alpha F}$ promoter in other
methylotrophic and methanotrophic bacteria has high homology to that of *M.*
extorquens. Accordingly, it is expected that other methanotrophic and methylotrophic
30 host cells will be suitable for the methods described herein.

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While other repressor/operator systems may be effective, in this case the regulatory elements of the *P. putida* F1 *cym* and *cmt* operons have been adapted, thus enabling inducible/regulated gene expression.

- 5 The metabolism of *p*-cymene (*p*-isopropyltoluene) by *P. putida* F1 is tightly regulated (21, 22). *P. putida* F1 degrades *p*-cymene (*p*-isopropyltoluene) through *p*-cumate (*p*-isopropylbenzoate) to isobutyrate, pyruvate, and acetyl coenzyme A. The genes encoding the enzymes required for this degradation are grouped in two distinct operons. The *cym* operon encodes the conversion of *p*-cymene to *p*-cumate, and
10 located downstream of the *cym* operon is the *cmt* operon which encodes the catabolism of *p*-cumate. A regulatory protein known as *cymR*, encoded upstream of the *cym* operon, has been shown to bind to specific operator-promoter regions in both operons and controls expression of both operons. Induction is afforded by *p*-cumate, the end product of the first operon, but not by *p*-cymene. Accordingly, the *cymR* gene
15 and the operator fragment of the *cmt* operon were chosen for the construction of the inducible expression system for *M. extorquens*.

- The construction strategy of the system is as follows. First, two copies of the gene encoding for the repressor protein *cymR* are integrated into the chromosome of *M.*
20 *extorquens* under the control of the strong native *P_{mxnF}* promoter. This promoter is, in practical terms, constitutive in *M. extorquens*. This recombinant repressor-expressing strain, *M. extorquens* (*cymR2*), is the host strain for subsequent plasmid transformations. Chromosomal integration of the *cymR* genes was achieved using the mini-Tn7 transposon system technology recently described in
25 Choi Y.J. et al, Appl. Environ. Microbiol., Jan. 2006, 72(1): 753-759.

- Second, an expression vector (*pCUM*-gene of interest) is constructed containing the strong native *P_{mxnF}* promoter, followed by the *cymR* binding site (operator sequence), and followed by the gene of interest to be expressed. This expression vector is then
30 cloned in the *M. extorquens* (*cymR2*). In the absence of the inducer, cumate, gene expression is repressed. Addition of cumate to the culture reduces the binding capacity of the repressor protein (*cymR*) to the operator region. Transcription repression is thereby alleviated and tight inducible expression of the reporter gene is attained.

The basic mechanism of this system is summarized in **Figure 1**. To test cumate-inducibility, we expressed the green fluorescent protein (GFP) and two other selected proteins (esterase, estI and β -galactosidase, bgl) in the recombinant host strain *M.*

5 *extorquens* (cymR2).

The development of an inducible/regulated gene expression system is an important addition to the *M. extorquens*' expression "tool box". Possible applications include the commercial expression of recombinant proteins, including that of toxic or
10 difficult-to-express proteins. This new tool permits modulated expression capability which may become useful when attempting to express potentially difficult or toxic proteins to the host. This tool may also be very useful in metabolic flux studies and methabolic pathway engineering.

15 MATERIALS AND METHODS

Bacterial strains and growth conditions The bacterial strains and plasmids used in this invention are listed in Table 1.

20 Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>M. extorquens</i> strains		
ATCC 55366	Wild-type	ATCC
CymR1	Modified host strain containing single copy of <i>cymR</i> expression cassette in the chromosome	This study
CymR2	Modified host strain containing double copy of <i>cymR</i> expression cassette in the chromosome	This study
GFP	<i>M. extorquens</i> CymR2 containing pCUM-gfp	This study
BGL	<i>M. extorquens</i> CymR2 containing pCUM-bgl	This study
EST	<i>M. extorquens</i> CymR2 containing pCUM-est	This study
<i>Pseudomonas putida</i> F1	Source of <i>cymR</i> gene in the <i>cym</i> operon	22
<i>E. coli</i> strains		
Top10	Strain for cloning and propagating plasmid DNA	Invitrogen Inc.
S-17/ λ pir	Host strain for pUX-BF13	23
Plasmids		
pBK-miniTn7- Ω Sm2	pUC19-based delivery plasmid for a miniTn7-Km transposon; Km ^r , Sm ^r	24
pBRI-tet	pUC19-based delivery plasmid for a miniTn7 transposon; Tet ^r	This study
pBRI80	pUC19-based delivery plasmid for a miniTn7	This study

pBRI-cymR1	transposon containing P_{mxnF} and RBS; Tet ^r pBRI80 plasmid containing one copy of cymR expression cassette	This study
pBRI-cymR2	pBRI80 plasmid containing two copies of cymR expression cassettes	This study
pUX-BF13	R6K replicon based helper plasmid	23
pCR2.1-TOPO	PCR cloning vector	Invitrogen Inc.
pCR-cymR	pCR2.1-TOPO plasmid containing <i>cymR</i>	This study
PCR-MDHOP	pCR2.1-TOPO plasmid containing P_{mxnF} -operator	This study
pCR-bgl	pCR2.1-TOPO plasmid containing <i>bgl</i>	This study
pCR-est	pCR2.1-TOPO plasmid containing <i>estI</i>	This study
pCR-gfp	pCR2.1-TOPO plasmid containing <i>gfp</i>	This study
pCM110	Wide-host range cloning vector containing P_{mxnF} ; Tet ^r	25
pCHOI3	Wide-host range cloning vector containing P_{mxnF} ; Km ^r	This study
pCUM50	Newly constructed regulative expression vector for <i>M. extorquens</i> (cymR+)	This study
pCUM-bgl	pCUM50 plasmid containing lactase expression cassette	This study
pCUM-est	pCUM50 plasmid containing esterase expression cassette	This study
pCUM-gfp	pCUM50 plasmid containing gfp expression cassette	This study
pUC19	Multi-purpose cloning vector	Invitrogen Inc.
pCESTa	Esterase gene source	17
pEBIG4	Lactase gene source	26
pQBI63	GFP gene source	Qbiogene Inc.

M. extorquens (ATCC 55366) was used for the preparation of the heterologous gene expression host. *E. coli* strain Top10 was used for cloning and propagation of recombinant DNA and S17-1 λ *pir* (*recA*, *thi*, *pro*, *hsdR*⁻*M*⁺ RP4:2-Tc:Mu:Km Tn7, λ *pir* lysogen; Sm^R, Tp^R) was used for propagation of the helper plasmid (23). *E. coli* was cultured in Luria Bertani broth (LB) at 37°C. Strain of *M. extorquens* was grown in CHOI medium, as previously described (17) and 1 % (v/v) methanol was used as sole carbon source. Both media were solidified by 1.8% agar (Difco) when appropriate. Antibiotics were used for *E. coli* and *M. extorquens* at the following concentrations (in ug/ml): ampicillin, 100; kanamycin (Km), 40; tetracyclin (Tc), 35.

Construction of expression host strains The expression host *M. extorquens* (cymR⁺) was constructed by insertion of the *cymR* gene of *P. putida* F1 using the mini-Tn7 integration system using the mini-Tn7 vector pBRI80 with helper plasmid (23, 24). Primers used in the *cymR* amplification were designed on the basis of the nucleotide sequence of *P. putida* F1 *cym* operon (22). In order to achieve tight regulated induction, two copies of *cymR* gene were integrated into the *M. extorquens* chromosome. Briefly, the mini-Tn7 base vector pBRI80 was constructed as follows: a 1,955-bp *Pst*I fragment containing TetA and TetR was amplified from pCM110 (25) by PCR using primers Tet-F-Pst (5'-GCTGCAGTCAATCGTCACCCTTTCTCGGTC-3') (*Pst*I site is underlined) and Tet-R-Pst (5'-GCTGCAGTCAGCGATCGGCTCGTTGCCCTG-3') (*Pst*I site is underlined). This fragment was then replaced with a kanamycin resistant gene in the pBK-miniTn7- Ω Sm2 to form pBRI-tet.

The P_{*mxoF*}-RBS was amplified from pCESTc using primers MDH-F-*Pst*I (5'-GGCTGCAGGTTGACGACAACGGTGCGATG-3') (*Pst*I site is underlined) and MDH-R-*Mlu*I (5'-CCGACGCGTATGTATATCTCCTTCTTAAAG-3') (*Mlu*I site is underlined). The PCR fragment containing P_{*mxoF*}-RBS was cloned into pBRI-tet which was partially digested with *Pst*I/*Mlu*I to delete Sm^R/Sp^R cassette, to generate pBRI80.

To generate pBRI-cymR1, *cymR* was amplified from chromosomal DNA of *P. putida* F1 using primers CYM-F-Afl (5' –GCTTAAGAAGATGGTGATCATGAGT CCAAAGAGAAGAAC –3') (*Afl*III site is underlined) and CYM-R-Not (5' –
 5 CAGCGGCCGCCTAGCGCTTGAATTTCG CGTACCGCTCTCGCG –3') (*Not*I site is underlined). A 612-bp *Afl*III-*Not*I fragment from pCR-cymR was ligated into the *Afl*III-*Not*I site of pBRI80 to form pBRI-cymR1. To obtain the pBRI-cymR2 containing two copies of *cymR* expression cassettes, a second copy of *cymR* was amplified from pBRI-cymR using primers MDH-CYM-F-Not (5' – CAGCGGCCGCCTTGACGACAAC
 10 GGTGCGATGGGTC –3') (*Not*I site is underlined) and CYM-R-Apa (5' –CAGGGG CCCCTAGCGCTTGAATTT CGCGTACCGCTCTCGCG –3') (*Apa*I site is underlined). Amplified fragment containing P_{mx}aF-RBS-*cymR* was then ligated into the *Not*I-*Apa*I site of pBRI-cymR1 to generate pBRI-cymR2.

15 The genotypes of the *cymR*-integrated host strains were confirmed by Southern hybridization using the 612-bp *cymR* fragment as a probe. Since we have identified the specific Tn7 insertion site (*att*Tn7) in *M. extorquens* in a previous study (23), the integration of target gene in the chromosome of *M. extorquens* was also determined by colony PCR using designed primers which include common Tn7 primers (P_{Tn7R}F; 5' –
 20 ATTAGCTTACGACGCTACACCC –3' and P_{Tn7L}R; 5' – CACAGCA TAACTGG ACTGATTTC –3') and gene specific primers (P_{dhaT}F; 5' – CATCGCGATTG TCGATTTCGG –3' and P_{glnS}R; 5' – CTGAAGGAAATCAGCTACATC –3'). The *cymR* positive strains were finally confirmed by Western blotting. Then, the electro-competent cells were prepared using *cymR* positive *M. extorquens*, essentially as described (6, 17).

25 **Inducible expression vector construction.** Manipulations and sequencing of DNA were carried out using standard procedures. The operator sequence of *cmt* operon from *P. putida* F1 was introduced downstream of the methanol dehydrogenase promoter, P_{mx}aF by polymerase chain reaction. The pCUM50 regulative expression vector was obtained in
 30 several steps: first, the P_{mx}aF –synthetic operator sequence was PCR-amplified from pCM110 using primers MDH-F-PST (5' – GCTGCAGGTCGACTCT AGATCA

CCTCCTTAAGC -3')(the *Pst*I site is underlined) and MDH-CUM-R (5'-CGAATTC
ATAATACAAACAGACCAGA TTGTCTGTTTGTG GCCCTTA
GGATCCGCGGTATC -3')(the *Eco*RI site is underlined). The 403 bp PCR fragment
containing *P_{mx}aF*-operator was cloned into pCR2.1 to create pCR-MDHOP. Next, the
kanamycin resistant gene of a 1,218 bp *Pst*I fragment from pBK-miniTn7-ΩSm2 was
cloned into the *Pst*I site of pCM110, then, *tet*A and *tet*R were removed by restriction
with *Bcl*I and self-ligation yielded pCHOI3. A 403 bp *Pst*I-*Eco*RI fragment from pCR-
MDHOP was then ligated between the *Pst*I-*Eco*RI sites of pCHOI3; this replaced the
P_{mx}aF with *P_{mx}aF*-operator to form the pCUM50 cumate inducible expression vector.

To test heterologous protein production using this cumate switch system, we obtained
*Xba*I-*Cla*I fragment containing *gfp* gene from pQBI63 and cloned into *Spe*I-*Cla*I sites of
pCUM50 to generate pCUM-gfp. The 2,100 bp fragment carrying the lactase gene (*bgl*)
from *Bifidobacterium infantis* was amplified from pEBIG4 (26) using primers BGL-F-
Nhe (5'- CGCTAGCGAACATAGAGCGTTCAAGTGGC -3')(the *Nhe*I site is
underlined)and BGL-R-*Cla* (5'- CATCGATTTACAGCTTGACGACGAGTACGCCG -
3') (the *Cla*I site is underlined). For the amplification of esterase gene (1,800 bp, *est*I)
from *Lactobacillus casei*, pCESTa (17) was used as a template with primers EST-F-Nhe
(5'- G GCTAGCGATCAATCTAAAACAAATC-3') (the *Nhe*I site is underlined) and
EST-R-*Cla* (5'- CATCGATTTATTTATTTGTAATACCGTCTGC-3') (the *Cla*I site is
underlined). These *Nhe*I-*Cla*I fragments of *bgl* and *est* were then replaced with a *gfp*
gene in the pCUM-gfp to form pCUM-bgl and pCUM-est, respectively. The different
proteins tested in the pCUM system were cloned via *Spe*I and *Cla*I (GFP) or via *Nhe*I and
*Cla*I (BGL and EST).

Detection of gene expression Detection of GFP was carried out by fluorescence
microscopy, and quantified by using a SPECTRAFluor Plus™ (TECAN Austria GmbH,
Grodig, Austria) under excitation and emission wavelengths of 485 and 508 nm,
respectively. Concentration of GFP was calculated based on a linear relationship between
concentration and fluorescence units determined using solutions of purified GFP

(Qbiogene). The biomass (X) was determined by cell dry weight measurement of the samples (Moisture Analyzer MA 30, Sartorius).

Esterase activity was determined by a spectrophotometric method using para-nitrophenyl caprylate (pNP-caprylate) as substrate. The rate of hydrolysis of pNP-caprylate at 37°C was measured in 50 mM sodium phosphate buffer (pH 7.0) according to the method described previously (27). One unit of activity was defined as the amount of enzyme that liberated 1 μ mol of p-nitrophenol per min under the given assay conditions. The β -galactosidase activity was measured with *o*-nitrophenol- β -D-galactoside (ONPG) as a substrate and one unit of activity was defined as the amount of enzyme that liberated 1 μ mol of *o*-nitrophenol per min (28). The protein concentration was estimated by the method of Bradford (29) using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

Western blotting. Integrative expression of repressor protein (cymR) was determined by western blotting using standard protocol. cymR was detected with rabbit anti-bCymR #422 antibody (0.1 g ml⁻¹) and a goat anti-rabbit IgG (H+L) HRP conjugate (0.1 μ g ml⁻¹; Pierce cat#31460, West Grove, PA). Cells were lysed in SDS-PAGE sample buffer.

1-Construction of the inducible expression system:

1a-Integrative expression of cymR in *M. extorquens*. First, the 612 bp *cymR* gene was cloned under the control of P_{mxoF} promoter, in the chromosome of *M. extorquens* using the Tn7 integration system. Our preliminary experiments showed that *M. extorquens* (cymR1) containing one copy of *cymR* gene, expressed repressor protein constitutively as expected. However, when the expression vector (pCUM-gfp) described under the heading “**Inducible expression vector construction**” was used to transform the recombinant host *M. extorquens* (cymR1) culture, only 80 % inhibition of reporter gene expression by cymR was achieved (data not shown). It was assumed that this incomplete repression was due to insufficient cymR production. In order to increase the level of cymR expression, a host strain was constructed containing two copies of *cymR* integrated in the chromosome of *M. extorquens*. This recombinant host, referred as *M.*

extorquens (cymR2), showed increased expression of repressor protein. When transformed with pCUM-gfp, GFP expression was almost completely blocked.

Insertion of the mini-Tn7- P_{mxoF} -cymR into the unique Tn7 attachment site of
5 *M. extorquens* was verified by colony PCR and confirmed by western blotting (**Fig. 2**).
The resulting strain, *M. extorquens* (cymR2), was used as a host strain for the expression
plasmids. In this experiment, the amount of biomass generated from modified *M.*
extorquens hosts (cymR1, cymR2) at the end of a batch fermentations (~48h) was almost
identical to the native strain (~55 g dry mass per litre), which indicates that *cymR* gene
10 integration and dosage does not negatively affect the fermentation capability of *M.*
extorquens. (**Fig. 3**).

1b-Expression vectors and Induction profile of clones. To construct the inducible
expression vectors, the operator sequence of the *cmt* operon from *P. putida* F1 was
15 introduced downstream of a homogeneous promoter, P_{mxoF} . Plasmids, pCUM-gfp,
pCUM-est, and pCUM-bgl, containing a GFP, esterase and β -galactosidase expression
cassette, respectively, were constructed (**Fig. 4**). *M. extorquens* (cymR2), transformed
with these plasmids grew well for many generations under non-inducing conditions while
still retaining their capacity for reproducible, high-level synthesis of reporter proteins
20 when induced.

To test the optimal concentration of inducer, cumate, recombinant *M. extorquens*
(cymR2) transformed with pCUM-gfp was grown to mid-log phase (0.8-1.0 units at
OD₆₀₀) at 30°C followed by induction with cumate at different concentrations. Reporter
25 gene expression was detectable 4 h post induction. Cumate induction was very sensitive
since about 47 % of reporter gene expression was obtained with 1 μ g cumate/ml (**Fig. 5**).
No growth difference was observed with the cumate concentrations tested (1-30 μ g/ml),
indicating that cumate does not negatively affect cell metabolism. Optimum cumate
concentration for effective induction of reporter gene expression was 15 μ g/ml (ca. 0.1
30 mM).

To determine the optimal induction point (time of induction), stably transformed *M. extorquens* cells were induced at different stages of batch growth (OD₆₀₀ of 0.4, 0.6, 0.8, 1.0, and 1.2) with 15 µg cumate/ml. Cells induced at OD₆₀₀ of 0.8-1.0 generated maximal protein yields in terms of specific activity, which accounts for 20-50 % more protein production relative to the other induction points (**Fig. 6**).

To confirm induction profile of clones, three clones were selected from each selection plate of recombinant *M. extorquens* stably transformed with the following pCUM-delivatives, pCUM-GFP, pCUM-est, and pCUM-bgl. These clones were analyzed for induction profiles and basal expression levels. Clones cultured up to an OD₆₀₀ of 0.8-1.0 on CHOI medium containing kanamycin, did not produce detectable levels of recombinant proteins in the absence of cumate. When transformants were induced with cumate, expression of the recombinant protein was detected in all clones tested at 4 hours post induction. Production of GFP remained active even after 24 hours post induction with maximum expression at 30 hours post induction (**Fig. 7a**); recombinant protein expression was not detected in non-induced cells. Very similar results were obtained with pCUM-est and pCUM-bgl constructs (**Fig. 7b,c**).

Using this system, recombinant protein yields in the range of 2-6 g/l were obtained under regulative conditions; more precisely, 6.1 g/l GFP (**Fig. 7a**), and $5,637 \pm 320$ and $6,742 \pm 480$ U of r esterase and β-galactosidase, respectively (**Fig. 7b,c**).

It is understood that the examples described above in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

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We claim:

1. A polynucleotide construct for use in association with a methylotrophic or
methanotrophic host cell, the host cell being capable of expressing a cymR
5 repressor protein exhibiting repressor activity which is regulatable by the addition
of an external agent, and the polynucleotide construct comprising a constitutive
promoter native to the host cell, wherein the promoter is fused to a cymR operator
sequence which is operatively linked to at least one gene of interest, wherein the
cymR operator sequence is in operative association with the cymR repressor
10 protein and wherein addition of the external agent upregulates expression of the at
least one gene of interest.
2. A polynucleotide construct as claimed in claim 1 wherein the cymR repressor
protein is derived from *Pseudomonas putida*.
15
3. A polynucleotide construct as claimed in claim 2 wherein the cymR operator
sequence is at least a portion of the *cmt* operon of *Pseudomonas putida*.
4. A polynucleotide construct as claimed in claim 1 wherein the external agent is
20 cumate.
5. A polynucleotide construct as claimed in claim 1 wherein the host cell comprises
a chromosome, and wherein at least one polynucleotide sequence encoding the
cymR repressor protein is integrated into the chromosome.
25
6. A polynucleotide construct as claimed in claim 1 wherein the promoter is λ or
 $P_{\text{mx}aF}$.
7. A polynucleotide construct as claimed in claim 1 wherein the at least one gene of
30 interest encodes a reporter protein.

8. A polynucleotide construct as claimed in claim 1 wherein the at least one gene of interest encodes a protein that is toxic or difficult to express in the host cell.
9. A polynucleotide construct as claimed in claim 7 wherein the reporter protein is selected from the group consisting of β -galactosidase, esterase and green fluorescence protein.
10. A polynucleotide construct as claimed in claim 1, further defined as a vector.
11. The polynucleotide construct of claim 10, wherein the vector is a viral vector or a plasmid vector.
12. A methylotrophic or methanotrophic prokaryotic host cell comprising at least one polynucleotide sequence that encodes a cymR repressor protein exhibiting repressor activity which is regulatable by the addition of an external agent, and further comprising a polynucleotide construct which comprises a constitutive promoter native to the host cell, wherein the promoter is fused to a cymR operator sequence which is operatively linked to at least one gene of interest, wherein the cymR operator sequence is in operative association with the cymR repressor protein and wherein addition of the external agent upregulates expression of the at least one gene of interest.
13. A host cell as claimed in claim 12 which belongs to the genus *Methylobacterium*.
14. A host cell as claimed in claim 13 which belongs to the species *Methylobacterium extorquens*.
15. A host cell as claimed in claim 14 which belongs to the species identified as *Methylobacterium extorquens* ATCC55366.

16. A host cell as claimed in claim 12 wherein the cymR repressor protein is derived from *Pseudomonas putida*.
17. A host cell as claimed in claim 16 wherein the cymR operator sequence is at least a portion of the *cmt* operon of *Pseudomonas putida*.
18. A host cell as claimed in claim 12 wherein the external agent is cumate.
19. A host cell as claimed in claim 12 wherein the promoter is P_{mxαF}.
20. A host cell as claimed in claim 12 wherein the at least one gene of interest comprises a gene that encodes a reporter protein.
21. A host cell as claimed in claim 20 wherein the reporter protein is selected from the group consisting of β-galactosidase, esterase and green fluorescence protein.
22. A host cell as claimed in claim 12 wherein the at least one gene of interest comprises a gene that encodes a recombinant protein.
23. A host cell as claimed in claim 12 having a chromosome, wherein the at least one polynucleotide sequence that encodes the cymR repressor protein is integrated into the chromosome.
24. A host cell as claimed in claim 23, wherein the at least one polynucleotide sequence that encodes the cymR repressor protein is inserted into the host cell chromosome at the mini-Tn7 transposon site.
25. A gene expression system comprising a host cell as claimed in claim 12.
26. A method for selectively controlling the expression of at least one gene of interest comprising the steps of a) obtaining a host cell as claimed in claim 12 wherein

expression of the at least one gene of interest is repressed due to the repressor activity of the cymR repressor protein; and b) exposing the host cell to an external agent which inhibits the repressor activity and allows for expression of the at least one gene of interest.

5



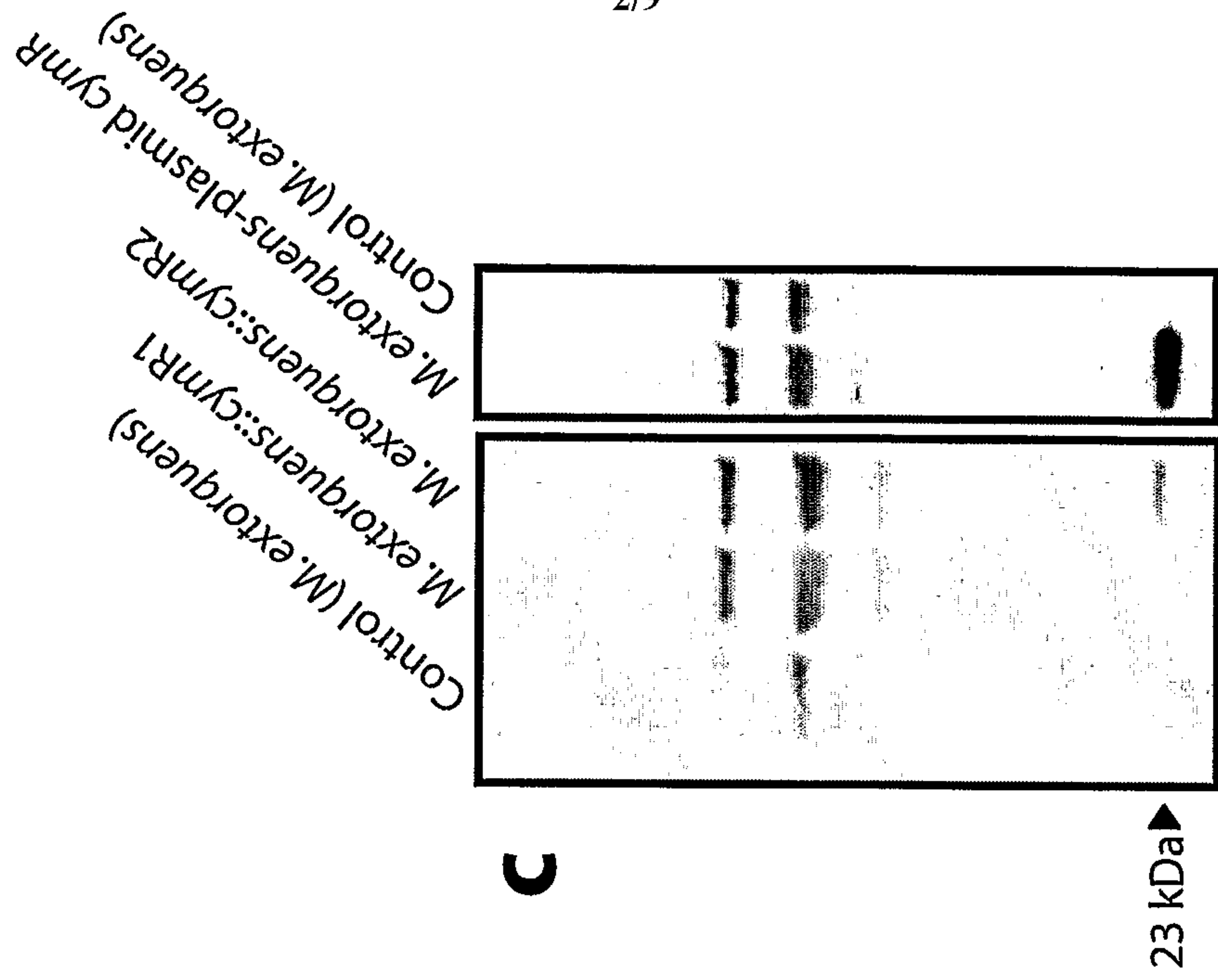
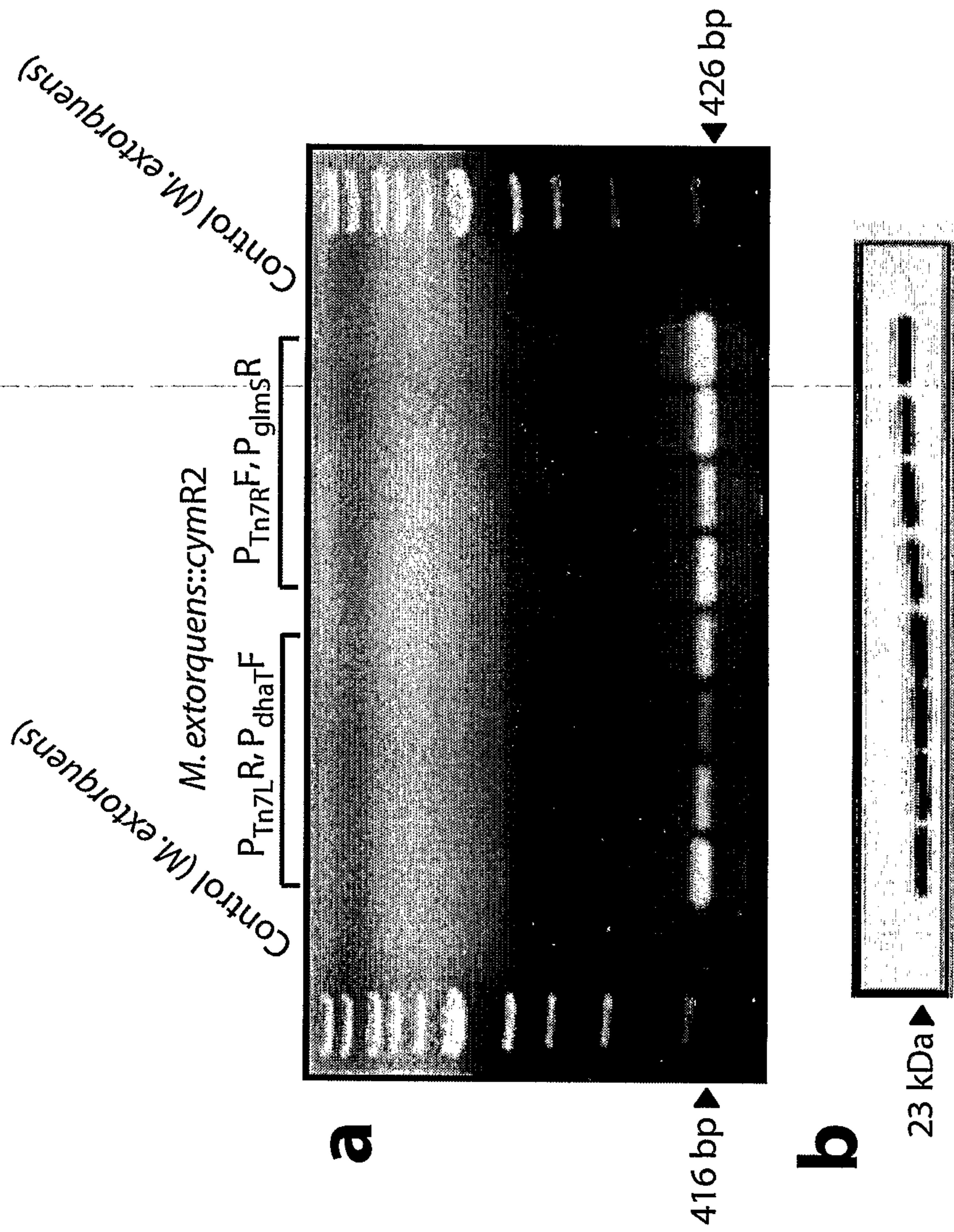
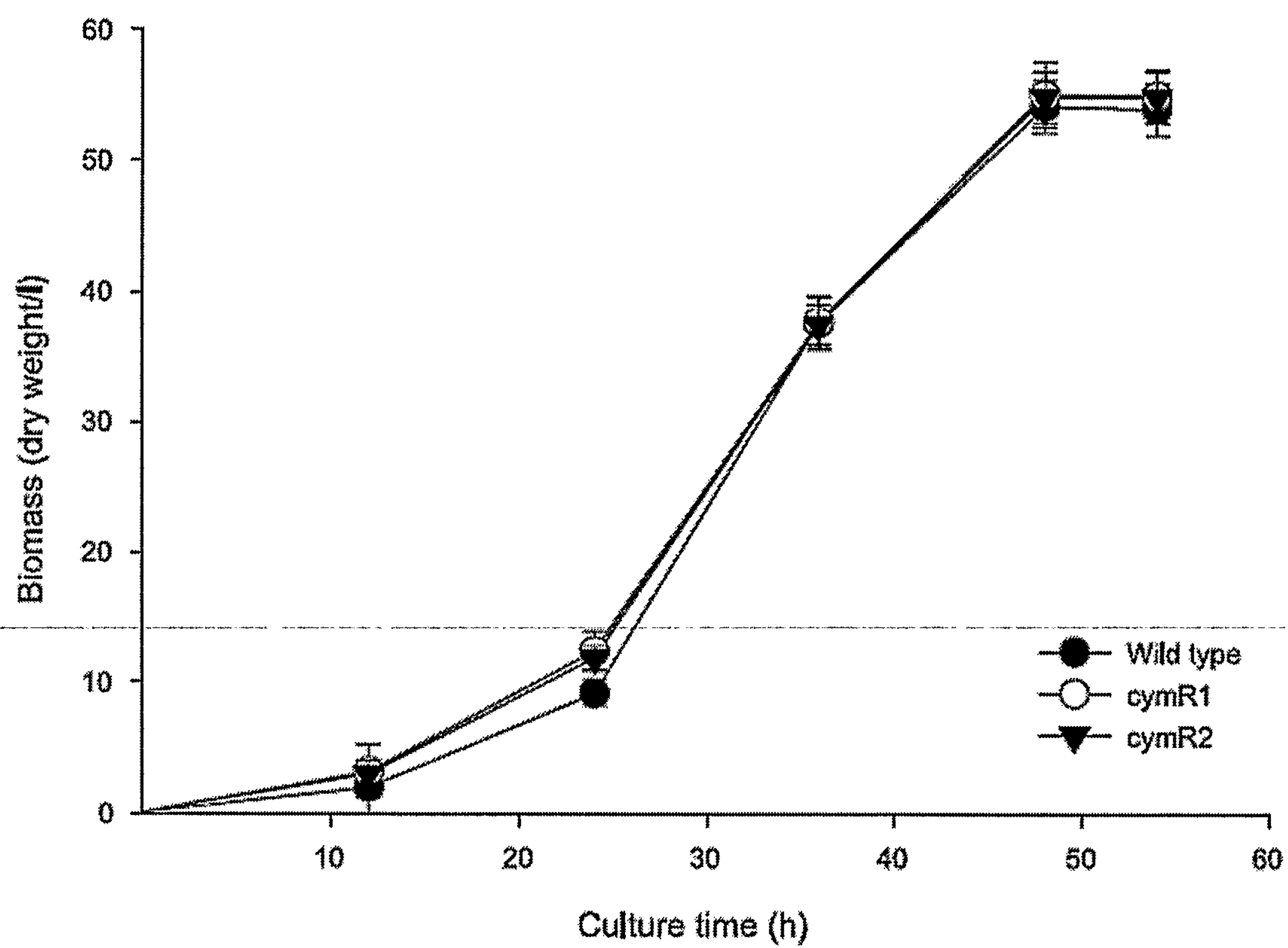
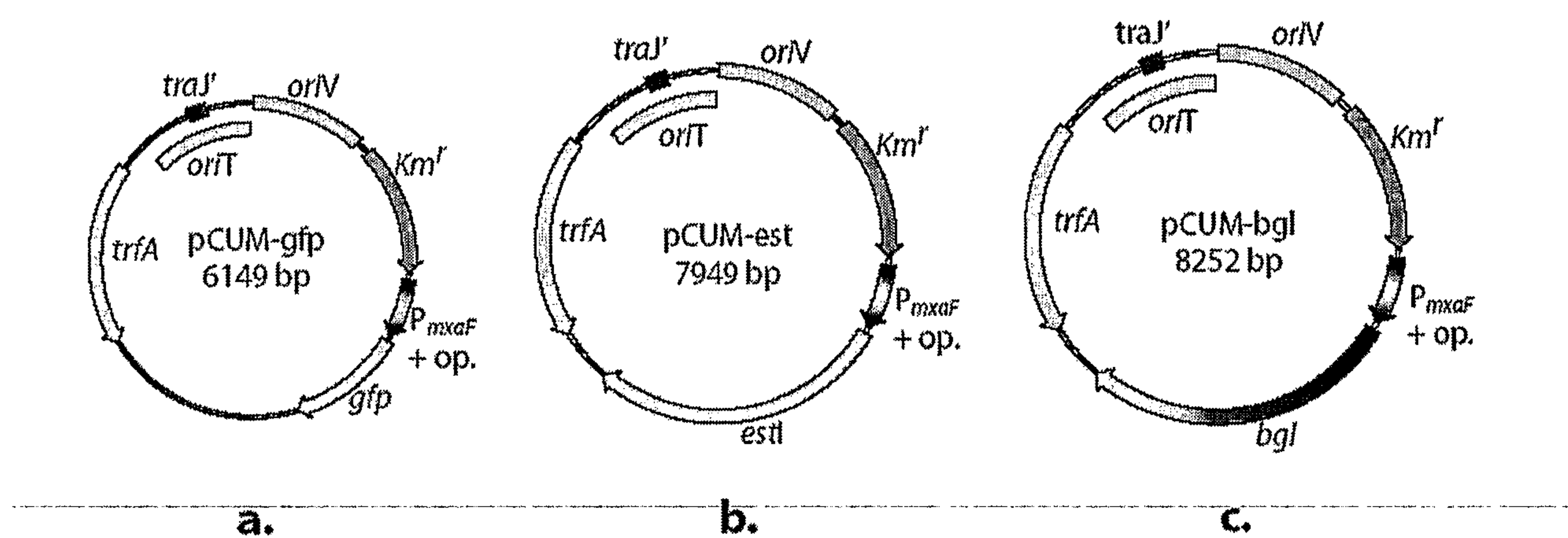


FIG. 2.

**Fig. 3.**

**Fig. 4.**

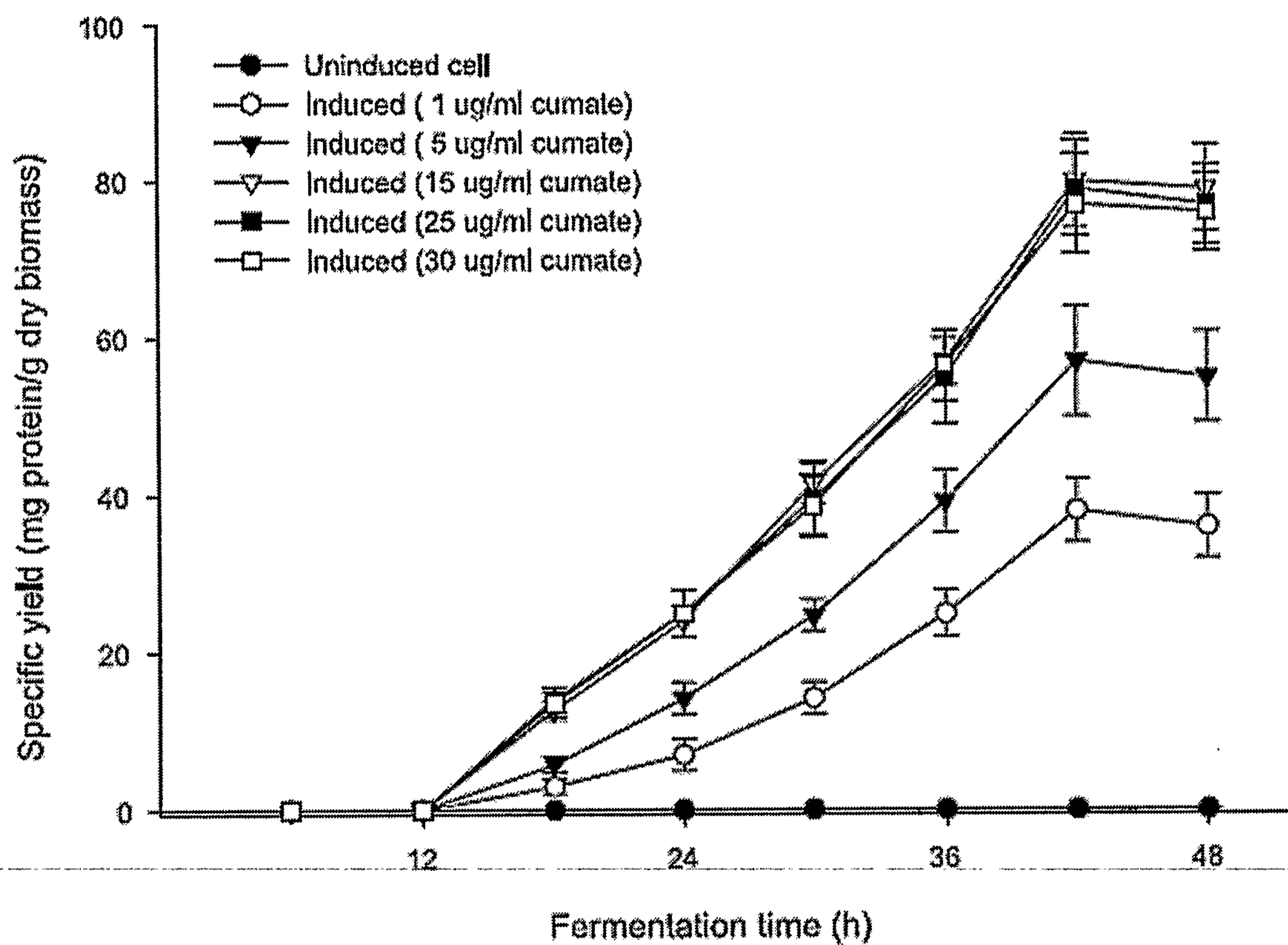
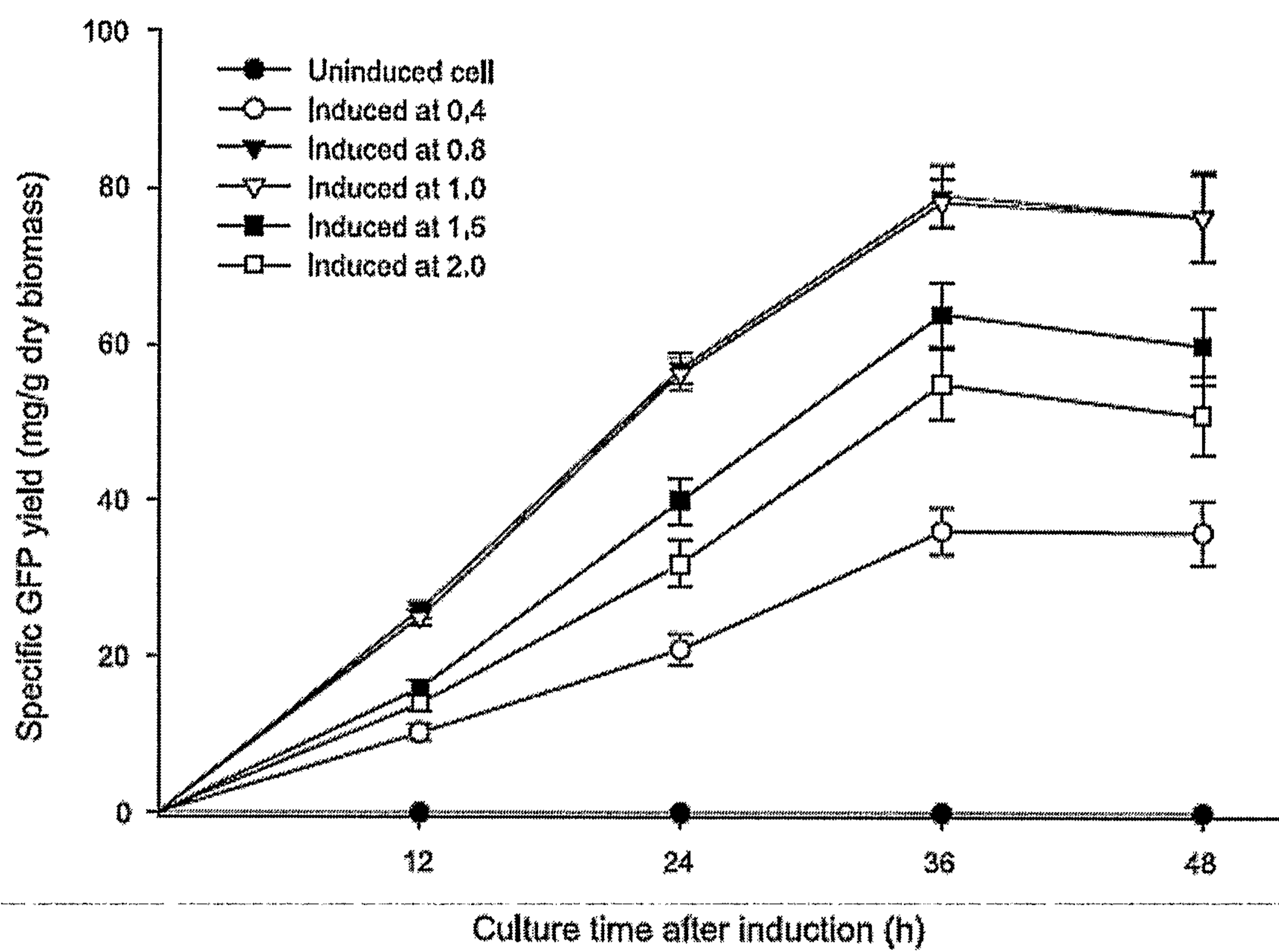
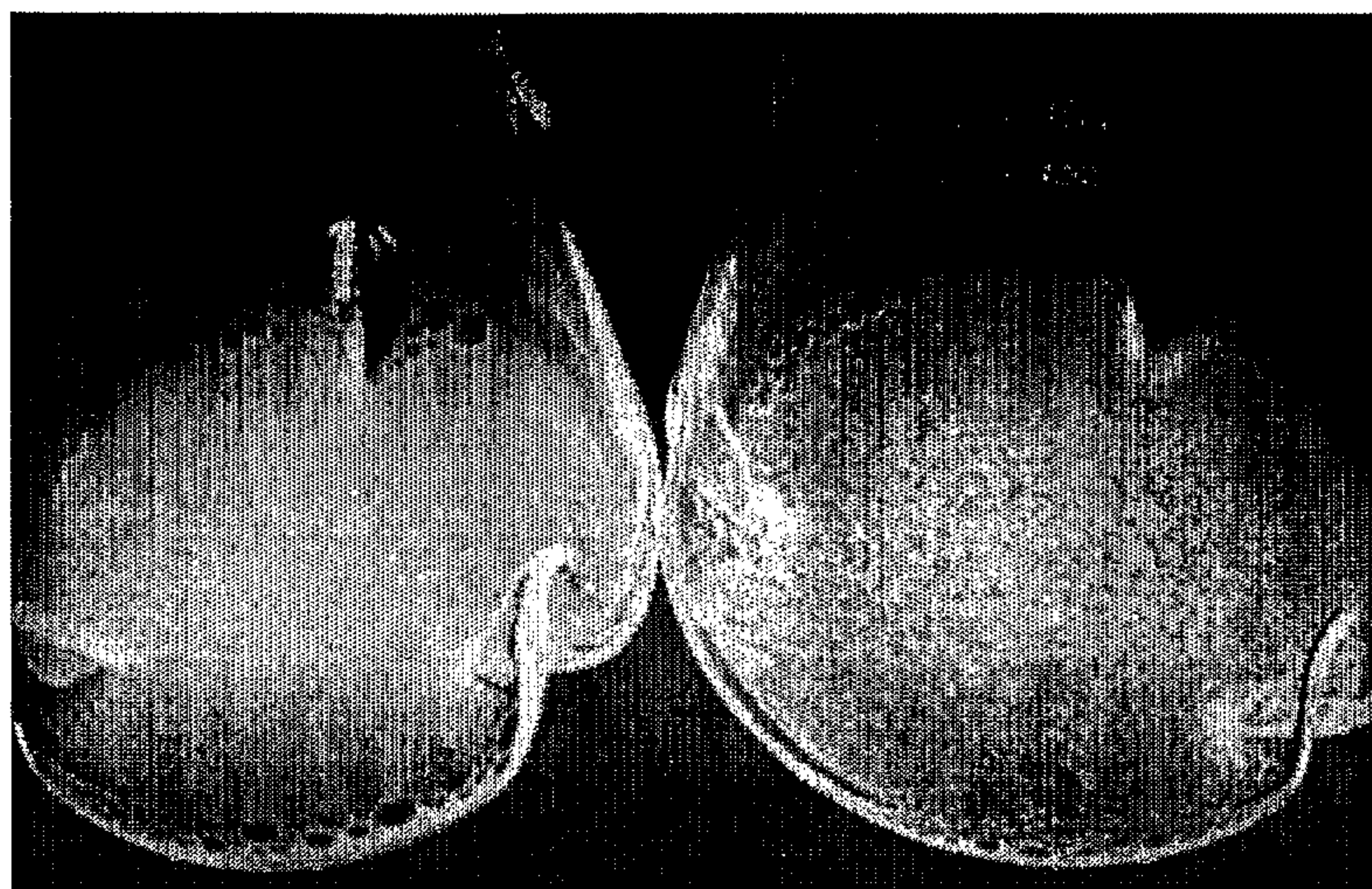


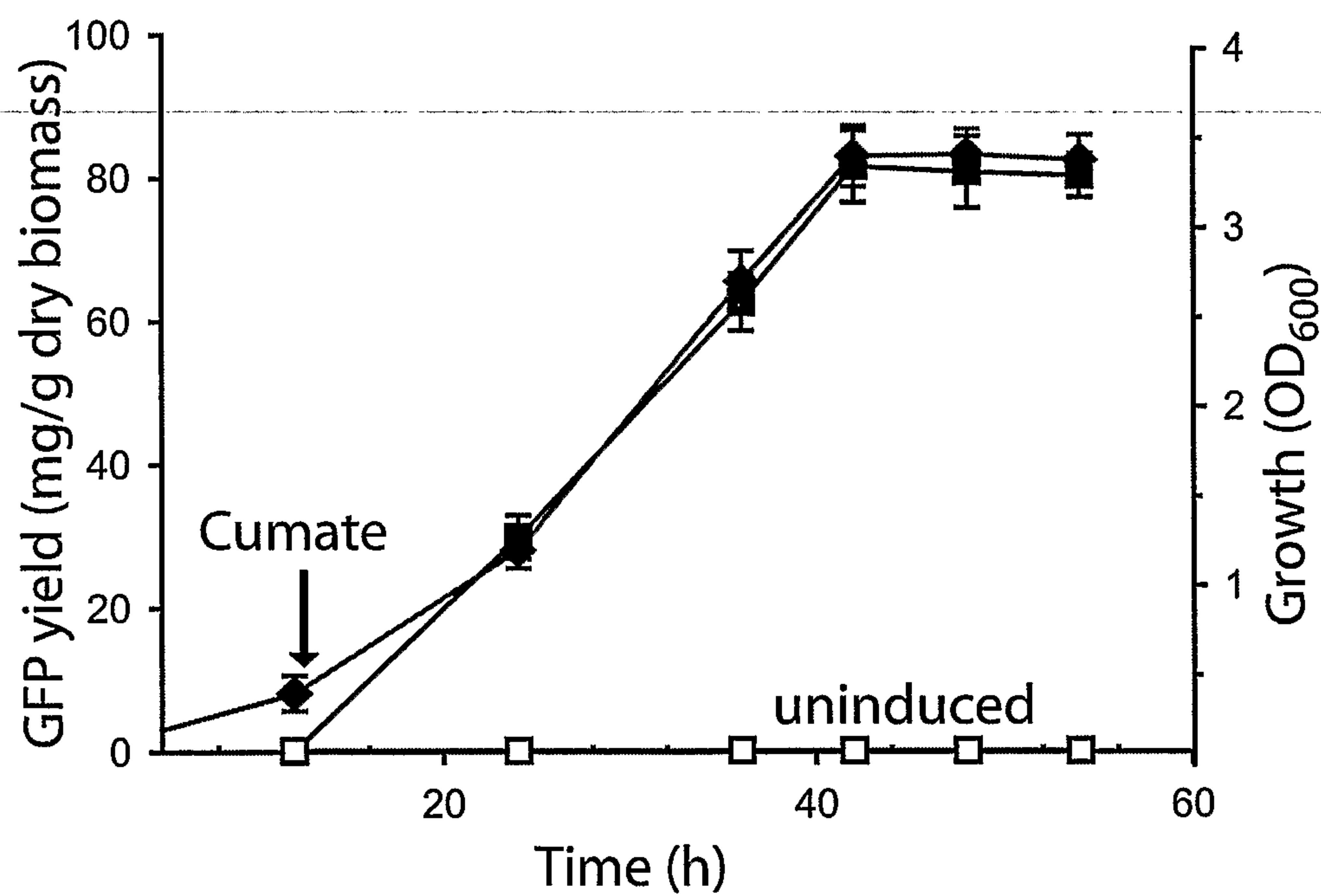
Fig. 5.

**Fig. 6.**

Uninduced Induced
(pCUM-gfp) (pCUM-gfp)



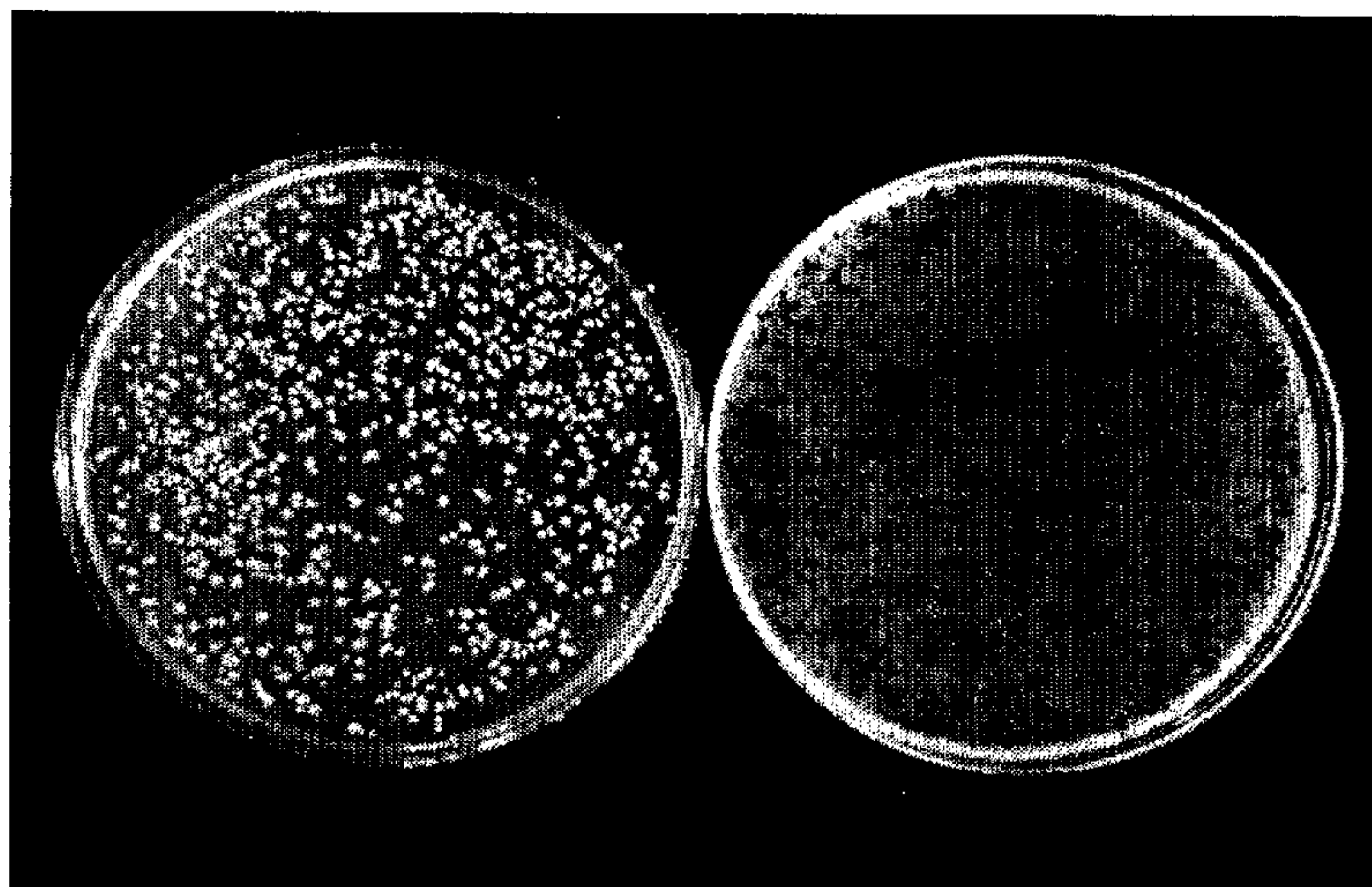
GFP



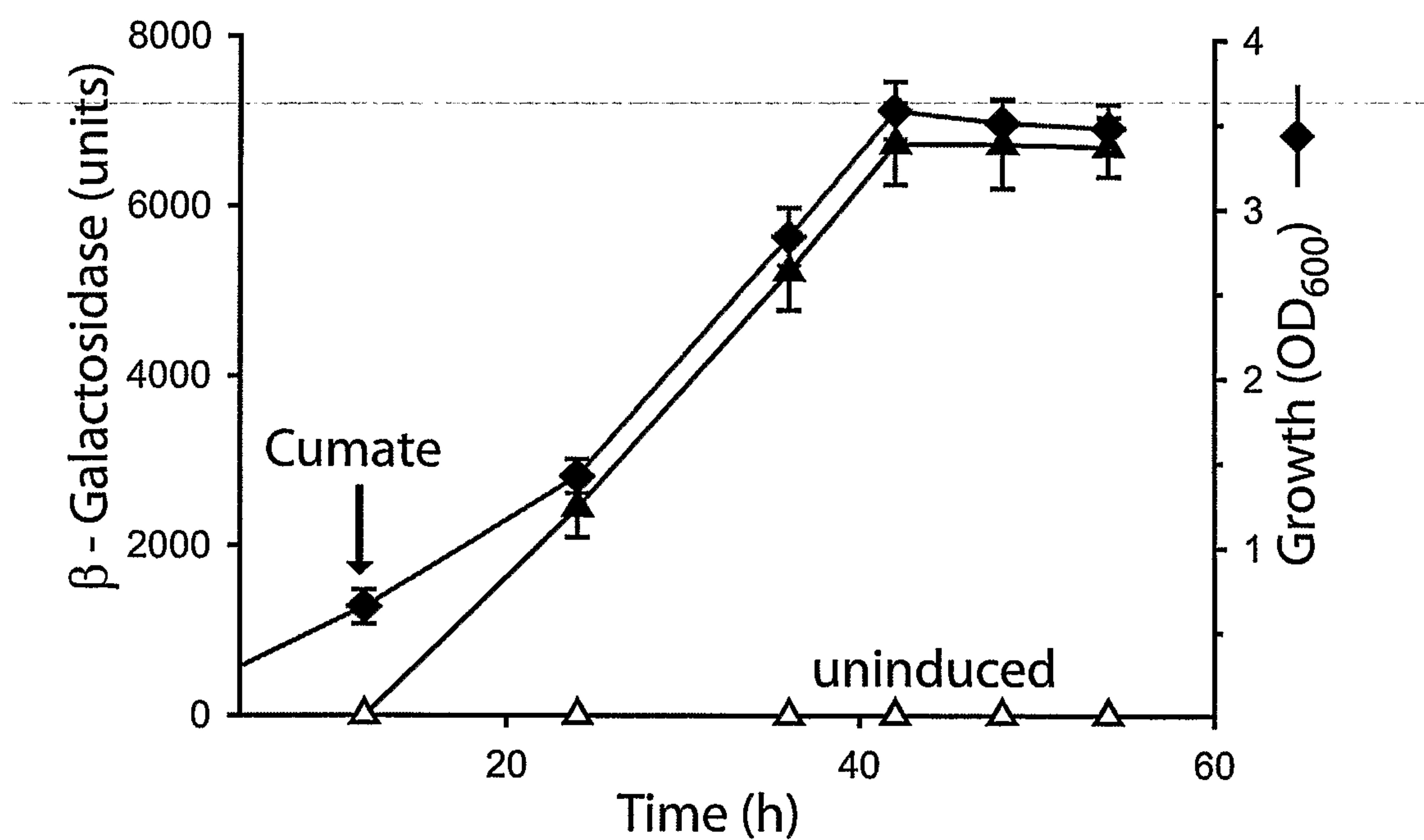
a

FIG. 7

Uninduced Induced
(pCUM-bgl) (pCUM-bgl)



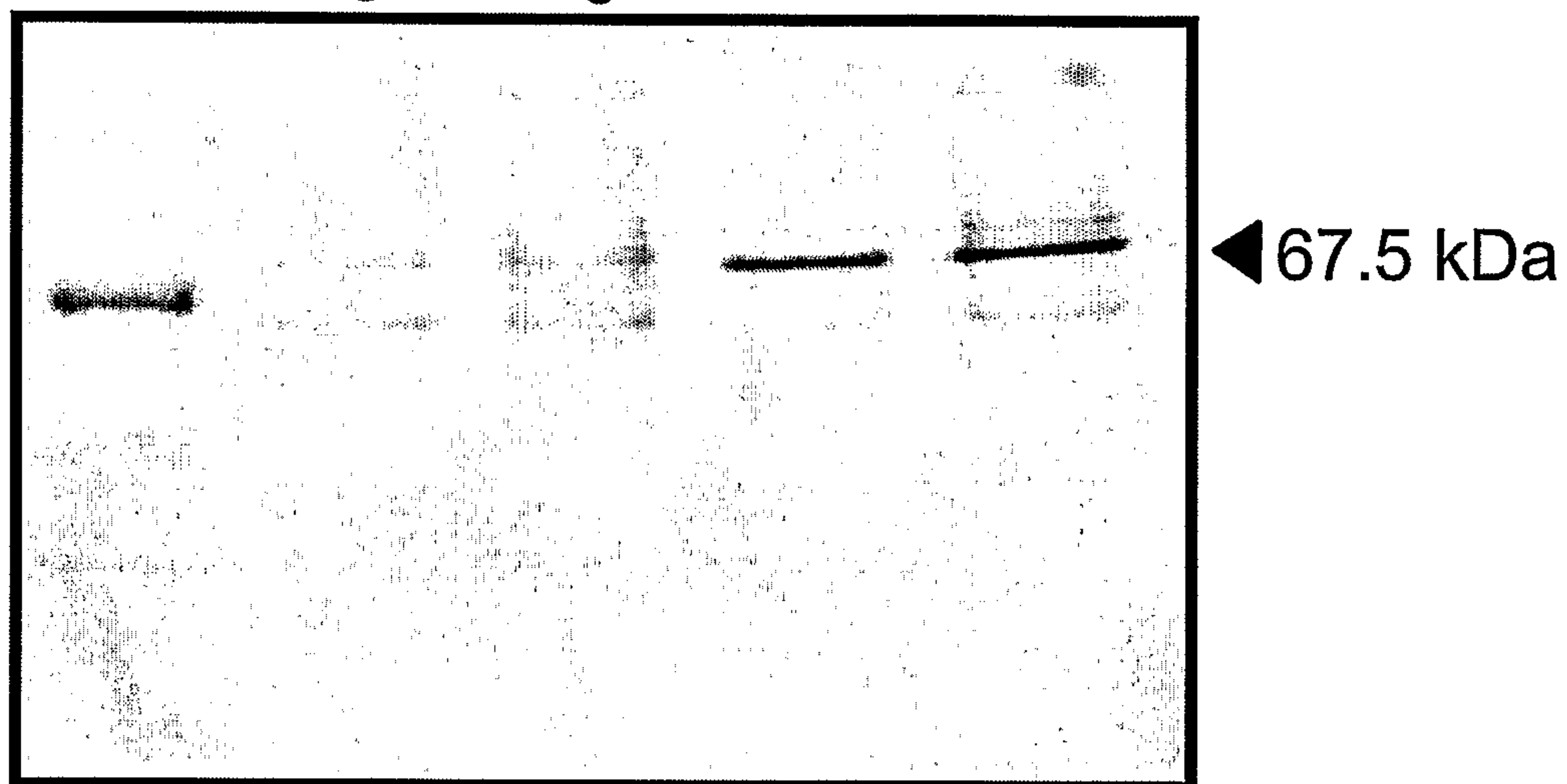
BGL



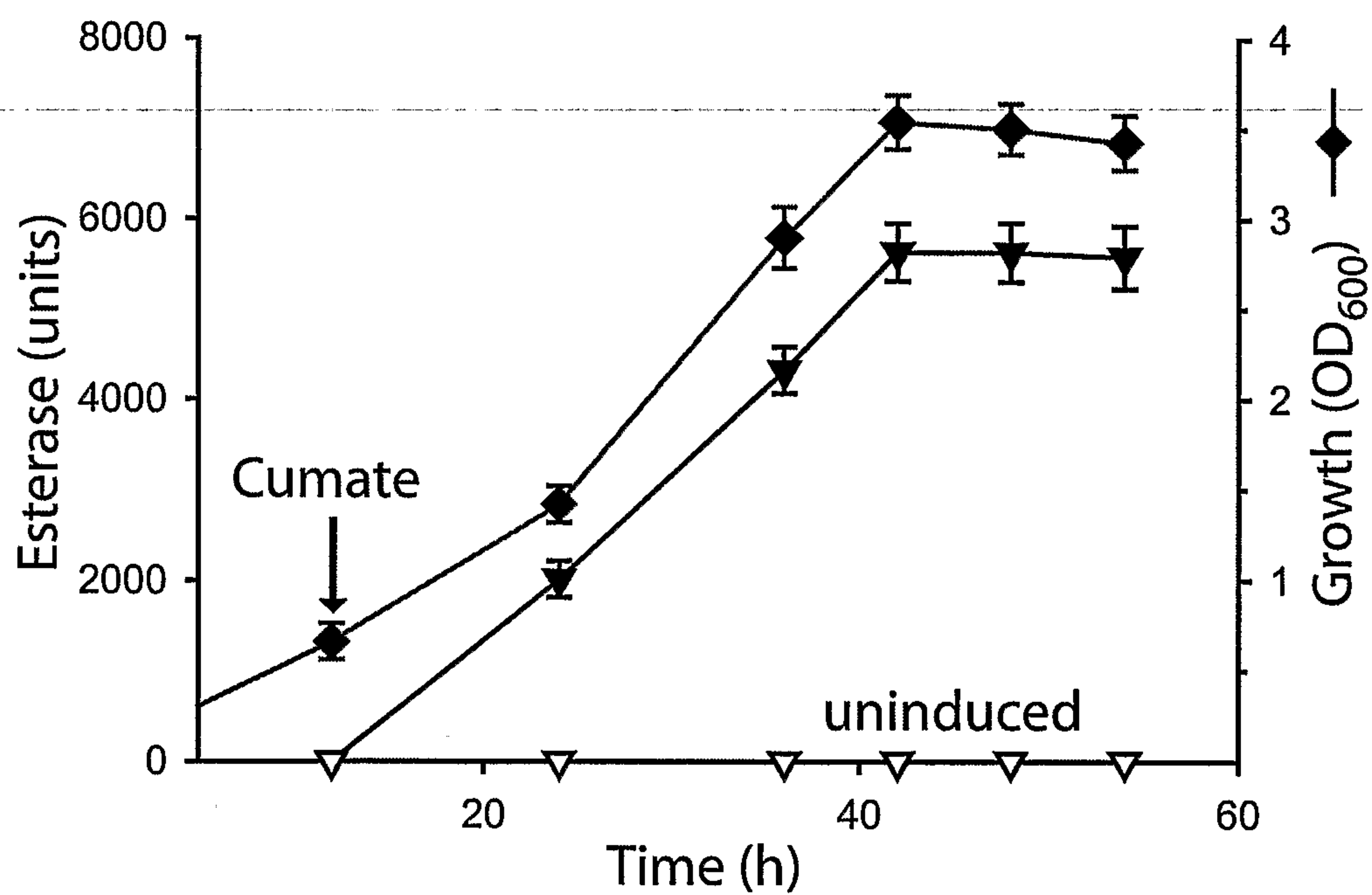
b

FIG. 7

Control (No plasmid)
Uninduced (pCUM-est)
6h after induction
12h after induction



EST



C

FIG. 7

