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(72) Inventeur/Inventor:
Grote, Mathias, DE

(73) Propriétaire/Owner:
HOECHST AKTIENGESELLSCHAFT, DE

(74) Agent: BERESKIN & PARR

(54) Titre : PROCEDE DE FERMENTATION OPTIMISEE, POUR LA PREPARATION DE PROTEINES ETRANGERES DANS E. COLI

(54) Title: OPTIMIZED FERMENTATION PROCESSES FOR THE PREPARATION OF FOREIGN PROTEINS IN E.COLI

(57) Abrégé/Abstract:
The invention describes optimized fermentation processes for the preparation of foreign proteins in E.coli using the lac promoter or improved lac promoter (for example tac, trc). After the initial growth phase with glucose as carbon source, induction of product formation is effected (1) via IPTG with glucose limitation or (2) via lactose with lactose limitation or (3) via IPTG and lactose with lactose limitation. The limitation of glucose or lactose is such that the oxygen partial pressure remains above 10%.

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BEHRINGWERKE AKTIENGESELLSCHAFT HOE 89/B 032 - Ma 768

Dr. Lp/rd

Abstract of the disclosure:

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BEHRINGWERKE AKTIENGESELLSCHAFT HOE 89/B 032 - Ma 768

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Description

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The invention describes optimized fermentation processes for the preparation of foreign proteins in E.coli using the lac promoter or improved lac promoter (for example tac, trc). After the initial growth phase with glucose as carbon source, induction of product formation is effected (1) via IPTG with glucose limitation or (2) via lactose or (3) via IPTG and lactose with lactose limitation. The limitation of glucose or lactose is such that the oxygen partial pressure remains above 10%.

The preparation of commercial quantities of many different recombinant proteins in E.coli is well known in principle. The expression of these proteins becomes possible by cloning the coding cDNA into a multicopy plasmid with the appropriate sequences.

Expression experiments on this are normally carried out in shaken flasks. The yields of recombinant proteins in this case are usually 50 - 100 mg/l when cultures in shaken flasks with volumes less than 100 ml are employed.

Although it is possible with these techniques successfully to prepare recombinant proteins, there is a need for techniques with which the protein concentrations and the preparable quantities are distinctly increased. One approach which meets these requirements is the development of a fermentation process. The object of the invention is consequently the optimization of fermentation processes for the expression of foreign proteins in E.coli.

Several such processes with which the said requirements have at least partially been met have been described in the literature. The use of the lac promoter in these cases has meant that usually fusion proteins with an N-terminal β-galactosidase portion (β-Gal) have been prepared. The yields in the fermentation are normally between 0.1 and 2.0 g of fusion protein per liter. When the fused β-Gal portion is taken into account, the actual product concentration often decreases to 30% of the said value. Furthermore, elaborate purification processes are required to remove the β-Gal portion from the product.

The present invention describes, inter alia, the expression of a mature product, i.e. the expression of a product without a fusion portion which would subsequently have to be eliminated again. Purification of the product is made relatively straightforward by such processes. However, in fermentation both the specific and the volumetric yields of the products are normally considerably lower. This is particularly true when the product of the process is prepared in soluble and biologically completely active form. In contrast to the formation of protein which is inactive and stored as inclusion bodies, the soluble and biologically active product may intervene in the metabolism of the cells and cause drastic disturbances in the organism (E.coli) and may be degraded considerably more easily by E.coli proteases. Despite these problems, it has been possible in the processes to date, in which glucose was employed as carbon source and isopropyl thiogalactoside (IPTG) was used for induction, to obtain yields of 200 mg/l of biologically completely active product.

In order to optimize the fermentation, the invention entailed improvements in the growth behavior and product formation. Since the product is formed within the cells, the specific product concentration (quantity of product per cell) and the cell count are important. The product of the two factors is the volumetric productivity of the

process in grams per liter (g/l).

High cell density fermentations have frequently been described in the literature for recombinant E.coli strains. Cell densities up to 30 g of dry matter (DM) per liter (l) are usually stated in this connection. It has been possible, by a combination of several measures which are known in principle, to develop a process in which the recombinant E.coli K12 strain was fermented up to cell densities of 50 g of DM/l, corresponding to 150 A_{650}. The essential point here is that oxygen-enrichment of the inlet air is not a condition of the process which is described hereinafter. This has a beneficial effect on the economics of the process because pure oxygen as substrate results in high costs and, additionally, it is possible to dispense with explosion-protection measures.

An important factor for a process with a high volumetric product yield is optimal induction of the promoter. Induction with IPTG, which is carried out with the abovementioned low cell densities, is described many times in the literature.

It has been found that an improvement in the volumetric yields by a factor of 5, from 0.2 g/l to 1.0 g/l, is achieved after induction by IPTG (1 mM to 10 mM, preferably 5 mM) and limitation of glucose as substrate in such a way that the oxygen partial pressure is greater than or equal to 10%.

A second embodiment of the invention comprises induction of product formation in the case of growth with lactose as carbon source and simultaneously natural inducer. The oxygen partial pressure was likewise maintained at greater than or equal to 10% as above by controlling the lactose addition. Induction by lactose is regarded in the literature as suboptimal because this procedure is alleged to be less efficient than IPTG induction. To date, no efficient fermentation processes in which

lactose was employed as inducer have been described. The experimental approach according to the invention is based on the consideration that a higher final concentration of product can be achieved by a weaker induction and thus slower product formation, because the slower product formation has a less disturbing effect on the intrinsic metabolism of E.coli. This approach has been confirmed in appropriate experiments in which product concentrations of 2 g/l (+/-10%) were achieved, corresponding to a doubling relative to above. The process differs from the previous one induced by IPTG in that glucose was replaced by lactose in the linear phase of growth. In the range of high metabolic activities at the end of the fermentation, when addition of lactose was also limited in order to maintain the partial pressure of oxygen above 10%, it was possible in a third variant of the process to assist the induction by lactose additionally by IPTG additions. This additional IPTG induction is necessary only when the power input of the specific chosen fermentation apparatus is inadequate to supply the culture with oxygen. Since an increased plasmid loss is observed in the second process described, on scale-up there is a crossing point as the fermentation volumes increase, after which first the second and then the first process is more economic, because a slightly increased plasmid loss is observed on induction with lactose.

The fermentation is terminated at the time when the product concentration is at a maximum. Processes known to those skilled in the art are used to concentrate (for example in a separator) and disrupt (for example in a high-pressure homogenizer) the biomass. After sedimentation of the cell fragments, most of the product is located in the clarified supernatant.

Accordingly, the invention relates to optimized fermentation processes for the expression of foreign genes in E.coli under the control of the lac promoter or optimized lac promoter, with induction being effected at the end of

the exponential phase of growth by

(1) IPTG with simultaneous substrate-limited glucose addition, and the oxygen partial pressure is maintained above 10% by the limitation of the glucose addition,

(2) or by lactose as carbon source and simultaneously natural inducer, with the oxygen partial pressure being maintained above 10% by the limitation of the lactose addition,

(3) or by lactose as carbon source and simultaneously natural inducer and, in addition, IPTG, with the oxygen partial pressure being maintained above 10% by the limitation of the lactose addition.

In preferred variants of the process, in each case the oxygen partial pressure is increased by one or more of the following measures:

(a) Fermentation under superatmospheric pressure, preferably up to 2 bar

(b) Controlled following of the power input (increasing the stirrer speed) and of the aeration rate (up to 2 vvm)

(c) Reducing the temperature from 37°C to as far as 30°C in order, via an improved Henry coefficient and reduced metabolic activity, to increase the oxygen transfer rate and reduce the oxygen uptake rate (necessary on scale-up above 1,000 l because the specific power input decreases with increasing batch size (= container)).

It is common to all the variants of the process that the addition of sugar substrate as carbon source is controlled to maximum values of 5 - 10 g/l and the pH is

controlled by addition of NH_4OH and H_3PO_4 in the range from pH 6.7 to pH 7.3 throughout the fermentation period.

The processes described above are preferably employed for the genetically engineered preparation of the proteins PP4 and PP4-x, which belong to the lipocortins, (Grundmann et al., Proc. Natl. Acad. Sci. 85, (1985) 3708-3712) and the mutants and variants thereof.

The invention is further described in the examples and patent claims.

Example:

The following example describes the fermentation of the E.coli K12 strain W3110 lac IQ (Brent and Ptashne (1981) Proc.Acad.Natl.Sci. USA 78, 4204-4208), this strain having been transformed with the plasmid pTrc99A-PP4 (Amann et al. (1988) Gene 69, 301-315) or pTrc99A-PP4-X (Grundmann et al. (1988) Behring Inst. Mitt. 82, 59-67).

Tab. 1 indicates a very suitable medium.

Tab. 1

Composition of an example of a growth medium;
(data in g or mg per liter)

Carbon source (sugar) as required		
Yeast extract	20	g
NaH_2PO_4 x H_2O	1.2	g
Na_2HPO_4 x 2 H_2O	8.5	g
KCl	1.0	g
$MgSO_4$ x 7 H_2O	2.0	g
Citric acid	0.25	g
NH_4Cl	5.0	g
Thiamine	5.0	mg
H_3BO_3	2.0	mg
$(NH_4)_6Mo_7O_{24}$x4 H_2O	0.8	mg
$CuSO_4$ x $5H_2O$	0.16	mg
KI	0.4	mg
$MnSO_4$ x 7 H_2O	2.02	mg
$ZnSO_4$ x 7 H_2O	1.6	mg

Fermentation was carried out in a 10 l Biostat E fermenter (manufacturer: Braun Melsungen) with a fermentation volume of 8 l.

During the fermentation no selection pressure was exerted on plasmid-containing cells, i.e. the fermentation was carried out without added antibiotics. The fermenter was inoculated with an overnight shaken-flask preculture. Glucose was employed as carbon source in the initial phase of growth, the glucose being metered in so as to form less than 0.1 M acetic acid in this phase. Increased acetate concentrations resulted in significantly lower product yields. After 10-15 hours in the initial phase of growth, cell concentrations of about 50 OD_{650} had been achieved, and induction of product formation was effected in three different alternative ways:

(1) Metering-in of glucose continued after addition of 1-10 mM IPTG (preferably 5 mM IPTG)

At the end of the initial phase of growth, the product formation was induced by adding 1 - 10 mM IPTG (preferably 5 mM IPTG) while continuing to meter in glucose as carbon source ("substrate"). In this case the rate of product formation showed a distinct dependence on the glucose concentration at the time. Glucose as actual substrate and IPTG as apparent substrate appear as competing substrates, with, according to the rules of diauxia, glucose partially or completely suppressing the activation of the lac operon. In this case yields of 1 g/l PP4 or PP4-X were attained in the glucose-limited system (glucose concentration less than 0.1 g/l).

The glucose limitation was carried out by setting the pump or by means of on-line HPLC measurement. The growth rate of the cells was not decreased by induction in non-limited systems, while the growth rate of the cells decreased as a function of the glucose concentration in limited systems as expected. Cell densities between 100 and 150 OD_{650} were reached, depending on the relevant growth rates.

(2) Metering in of lactose continued

At the end of the initial growth phase, the product formation was induced by replacing glucose by lactose as carbon source. Lactose is the physiological inducer of the lac operon, but it brings about less complete induction than IPTG. During the induction phase, the cells continued to grow to cell densities of 100 OD_{650}. The product concentration reached values of 1.5 g/l.

(3) Metering in of lactose continued, and additions of 1-10 mM IPTG (preferably 5 mM)

At the end of the initial growth phase, the product

formation was induced by replacing glucose by lactose as carbon source and additionally adding IPTG. In this case, strong induction is brought about by IPTG and, at the same time, the physiological substrate lactose is utilized. Accurate metering in of the carbon source is unnecessary in this case, in contrast to glucose + IPTG. An excess of up to 30 g/l lactose has no adverse effect on productivity. During induction the cells likewise continue to grow up to cell densities of 100 OD_{650}. The product concentration at the end of fermentation is 2.0 g/l.

The induction is carried out by one of the processes as a function of the particular fermentation batch size, because the plasmid stability decreases from (1) to (3).

The fermentation parameters chosen in the described experiments are summarized in Tab. 2.

Tab. 2: Fermentation parameters

pH:	7.0 (controlled by addition of H_3PO_4 and NH_4OH)
Aeration rate:	0.5 - 2.0 vvm
Number of revolutions:	1,500 rpm
Temperature:	37°C (to 30°C)
Gage pressure:	to 2.0 bar
Substrate concentration:	Glucose controlled at less than 5.0 g/l, limited when dissolved oxygen decreases; lactose controlled in subsequent metering in (less than 30 g/l), limited when dissolved oxygen decreases.
Dissolved oxygen:	greater than 10%

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

1. A process for the expression of a foreign protein in E. coli using a lac promoter inducible by lactose, which comprises, in the case of induction by IPTG and with glucose as carbon source, controlling the glucose concentration so that the oxygen partial pressure is greater than or equal to 10%, wherein the expression of the foreign protein is induced at the end of the exponential growth phase.

2. A process for the expression of a foreign protein in E. coli using a lac promoter inducible by lactose, which comprises, in the case where lactose is used as carbon source and natural inducer, controlling the lactose concentration so that the oxygen partial pressure is greater than or equal to 10%, wherein the expression of the foreign protein is induced at the end of the exponential growth phase.

3. A process for the expression of a foreign protein in E. coli using a lac promoter which is inducible by lactose, which comprises, in the case where lactose is used as carbon source and natural inducer and, in addition, IPTG is used as inducer, controlling the lactose concentration so that the oxygen partial pressure is greater than or equal to 10%, wherein the expression of the foreign protein is induced at the end of the exponential growth phase.

4. The process as claimed in claim 1, 2 or 3, wherein at least one of the following constituents of the process is applied.

(a) fermentation under superatmospheric pressure,

(b) controlled following of the power input (increasing the stirrer speed) and of the aeration rate (up to 2 vvm),

(c) reducing the temperature from 37°C to as far as 30°C,

(d) controlled addition of substrate as carbon source to maximum values of 5 to 10 g/l,

(e) controlling the pH to values between pH 6.7 and pH 7.3.

5. The process as claimed in claim 4 wherein the fermentation under superatmospheric pressure is up to 2 bar.

6. The process as claimed in claim 1, 2, 3, 4, or 5, wherein E. coli strains containing cDNA of lipocortins are fermented.

7. The process as claimed in claim 6, wherein the cDNA codes for PP4, PP4-x or mutants and variants of PP4.