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Process for using the yeast ADH II promoter system for the biotechnological production of heterologous proteins in high yields

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Abstract

Process for using the yeast ADH II promoter system for the biotechnological production of heterologous proteins in high yields

The invention relates to a novel process for using the yeast ADH II promoter system for the biotechnological production of heterologous proteins in high yields, for example for preparing hirudins, miniproinsulins, leptins or derivatives thereof.

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Invention Title: PROCESS FOR USING THE YEAST ADH II PROMOTER SYSTEM FOR THE BIOTECHNOLOGICAL PRODUCTION OF HETEROLOGOUS PROTEINS IN HIGH YIELDS

The following statement is a full description of this invention, including the best method of performing it known to us :-

Description

Process for using the yeast ADH II promoter system for the biotechnological production of heterologous proteins in high yields

The invention relates to a novel process for using the yeast ADH II promoter system for the biotechnological production of heterologous proteins in high yields.

The polypeptide hirudin, which was originally isolated from the leech Hirudo medicinalis, is a highly specific thrombin inhibitor possessing a broad therapeutic potential (F. Markwardt, Biomed. Biochim. Acta 44 (1985) 1007-1013). However, it is only possible to prepare the quantities which are required by the recombinant route using transformed microorganisms. In this context, it has been found that the yeast Saccharomyces cerevisiae is suitable as a host organism for producing hirudin which is correctly folded and fully active (EP A1 168 342, EP A1 200 655).

Hirudin is to be understood to mean peptide-like thrombin inhibitors which have a specific activity of at least 10,000 ATU/mg (anti-thrombin units/mg), which are derived from the known isohirudins of the species Hirudo medicinalis and which exhibit essential structural features of these isohirudins, in particular the characteristic linking of the three disulfide bridges (J. Dodt et al., Biol. Chem. Hoppe-Seyler 366 (1985) 379-385), (cf., for example, EP A1 158 564, EP A1 168 342, DE 34 45 517, EP A2 193 175, EP A1 200 655, EP A1 158 986, EP A1 209 061, DE 33 42 199, EP A1 171 024).

In particular, among these, a hirudin is understood to be a hirudin as described in EP A1 171 024, EP A1 158 986 and EP A1 209 061.

The gene for alcohol dehydrogenase isoenzyme II (ADH II) is strictly

regulated in yeast cells. The product of the ADH II gene is not found when fermentable carbon sources, such as glucose, are added to the fermentation medium.

Conditions for inducing the ADH II promoter can also be achieved in simple aerobic batch processes, in shaking flasks or in fermenters, which are started, for example, using a glucose concentration of 4%. When growth takes place on glucose, the so-called Crabtree effect results in ethanol being formed initially with the aid of the enzyme ADH I, with the ethanol in turn being used as an additional carbon source once the glucose has been completely consumed. After the glucose has been broken down, the ethanol-degrading enzyme ADH II is induced and the expression of hirudin begins.

As a rule, such batch fermentations do not provide the high space/time yield which is sought for industrial applications. What is wanted is the separation of the fermentation into a growth phase and a production phase, as is achieved in classical antibiotic fermentations, for example, by means of a limited fed-batch procedure, in which, after a certain cell density has been established, a carbon source is then fed in a growth-limiting manner in order to form the product (e.g. the secondary metabolite penicillin) at high yield under optimal physiological conditions (Hersbach et al.: The Penicillins: Properties, Biosynthesis and Fermentation, in: Biotechnology of Industrial Antibiotics, pp. 45-140, Ed. E.J. Vandamme, Marcel Dekker, New York, 1984).

Because of the way it is regulated, the ADH II promoter system is used for the biotechnological preparation of recombinant proteins in the bakers' yeast Saccharomyces cerevisiae (Price et al.: Methods of Enzymology, Vol. 185, 308-318, 1990). In this context, the fermentation is based, in dependence on the growth of the yeast cells, on the natural elimination of glucose as a carbon source. In the absence of glucose, product formation is switched on and continues until the end of the

fermentation. However, the yield of product appears to be less than was hoped.

Tøttrup et al. (Biotechnol. Bioeng., 35, 339-348, 1990) describe a modified fermentation procedure for the intracellular production of a human insulin fusion protein, which procedure involves feeding with glucose followed by feeding with ethanol in order to ensure optimal induction of the system. These workers succeeded in almost doubling the volume-related product yield, as compared with a batch process, by means of continually feeding glucose at a constant rate over the whole of the fermentation period. A further doubling was finally achieved by means of replacing the glucose feed, from a particular time point onward, with an ethanol feed. It was concluded from this result that even when there is no glucose, or very low glucose concentrations of < 20 mg/l, in the culture broth, the hybrid promoter is still partially repressed by glucose or a metabolite of glycolysis. Complete induction is only achieved by adding ethanol.

If the expression system described in the article by Price et al. is used for preparing the hirudin gene product mentioned in European Patent Specification 0 324 712 B1 (see Example 1), we have then observed, surprisingly, that the specific, biomass-based product yield decreases as a result of adding ethanol, continuously or discontinuously, since an increased cell growth can be observed in association with a product titer which is of the same magnitude.

The direct addition of ethanol has been investigated on a laboratory scale. While the additional supplementation of a batch culture with ethanol during growth on ethanol increased the biomass, it did not lead to any increase in product concentration as compared with the culture without supplementation.

Similarly, laboratory cultures which were carried out using ethanol as the

sole carbon source did not, within the limits of measurement accuracy, afford any specific yields (based on the optical density or on the biomass) which were higher than those of a comparison culture using glucose (see Ex. 2 as well). From this it may be concluded that ethanol on its own is not an inducer of ADH II.

Similarly, the attempt to generate derepressed growth, and to avoid ethanol formation due to the Crabtree effect, by means of regulated, model-assisted glucose feeding (following up, i.e. increasing, the feeding rate in accordance with the increasing cell density), in order then, in the 2nd stage, to induce expression more powerfully by means of a pulse addition of glucose with a subsequent high level of ethanol formation as a consequence of the Crabtree effect, did not result in any increase in product yield.

Surprisingly, a fermentation method which is simple and self-regulating and which, above all, can be carried out on an industrial scale has now been found for using the yeast ADH II promoter system for the biotechnological production of heterologous proteins in high yields. This process comprises continuously metering glucose, as the carbon source, into a fermentation culture (main culture) in which no glucose was initially present. During the course of the culture, various phases are passed through in such a manner that optimal induction and product formation are achieved. In the light of the state of the art, for example of the Price et al. literature reference which has already been cited, such an induction of heterologous gene expression in yeast using the ADH II promoter system is quite remarkable since Price et al. indicate that the ADH II promoter is only derepressed after there has been a marked depletion of glucose.

In our system, we were able to achieve an optimal product yield, associated with a high specific productivity, if a small quantity of glucose is fed in continuously at a constant rate directly after the main culture has

been inoculated. The increase in volume of the culture due to the feeding-in can in this case lead to a lowering, by up to 25%, of the actual glucose feeding-in rate by the time the culture has ended. At first, glucose is present in excess for the low cell population, i.e. with growth being repressed, ethanol is formed as a consequence of the Crabtree effect. From a particular time onward, the cell density is so high that glucose no longer suffices as sole carbon source and mixed growth on glucose and ethanol takes place. It is then only when the ethanol which was primarily formed has also been consumed and further (depressed) growth is limited by glucose that the ADH II promoter is switched on and product formation begins. In this way, the product yield can be approximately tripled as compared with the batch process.

The invention consequently relates to a process for the biotechnological fermentation of heterologous proteins in yeast in which a main culture is inoculated with a preliminary culture of a yeast strain which can express the heterologous protein, immediately after the inoculation a small quantity of glucose, e.g. 0.7-1.4, preferably 0.9-1.3, and very particularly preferably 1.1, g of glucose/l of culture volume/h is supplied continuously at a constant rate, aerobic culture conditions are ensured during the whole of the fermentation by not allowing the oxygen partial pressure to fall below 20% of saturation, the fermentation is terminated after 2 days and, finally, the heterologous protein is isolated from the fermentation culture.

Expression of the heterologous protein by the yeast strain may be achieved by inserting a heterologous gene under the control of an ADH II promoter into the genome of the yeast. Alternatively, and preferably in relation to the present invention, the yeast may contain an expression vector containing a gene encoding the heterologous protein.

The invention furthermore relates to a process in accordance with that described in the preceding paragraph in which the media of the preliminary culture and the main culture are equivalent apart from an addition of 1% glucose to the preliminary culture medium, which addition has preferably been almost

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completely consumed at the time of inoculation of the main culture, and contain one or more complex constituents, for example cornsteep, soyabean meal or yeast extract. In this context, "almost completely consumed" denotes a glucose

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concentration of < 100 mg/l.

The yeast strains used in said processes can be Saccharomyces cerevisiae, preferably Y79 (see EP 0 324 712) or else Kluyveromyces lactis strains.

The heterologous proteins which can be prepared in accordance with the process can be, for example, a hirudin, a miniproinsulin (as described, for example, in EP 0 347 781), a leptin (e.g. *obese* gene products as described in Zhang et al., Nature 372:425-32, 1994) or derivatives of the previously mentioned proteins or fusion proteins which contain the previously mentioned proteins and/or derivatives. In this context, the term derivatives denotes, inter alia, functional, i.e. biologically active, fragments of the starting proteins or mutants possessing novel, in particular advantageous, biological properties.

Example 1: Preparation of the expression vector for the recombinant production of hirudin in yeast

The vector pα ADH 2 (see Figure 2A in the paper by Price et al.) contains, adjacent to the Spe I restriction enzyme cleavage site in the direction of the 3' end of the cDNA, a recognition site for the enzyme Nco I which is unique for the plasmid.

If the DNA of the described vector is now reacted with Kpn I and Nco I, two DNA fragments are then obtained which are separated from each other by gel electrophoresis. The larger of the two fragments is isolated and reacted, in a T4 ligase reaction, with a Kpn I/Nco I hirudin fragment which is isolated from the plasmid Pαf Hir 17 (EP 0 324 712 B1) by partial digestion with the enzyme Kpn I and complete digestion with the enzyme Nco I.

Commercially obtainable competent E.coli K12 cells of the strain HB101

are transformed with the ligation mixture and the transformation culture is plated out on Na agar plates which contain 25 mg/l ampicillin. The plates are incubated at 37°C overnight and, on the following morning, individual colonies are picked from the plates and used to start overnight cultures. Plasmid DNA is in each case isolated from the cells of the cultures and examined by means of restriction analysis. Correct plasmid DNA, which contains the hirudin gene incorporated in the desired manner, is used to transform the Saccharomyces cerevisiae strain Y79 as described in EP 0 324 712 B1.

This gives the expression system which has been used for developing the described process.

Example 2: Comparison of laboratory cultures for the recombinant preparation of hirudin in yeast using different carbon sources

Different carbon sources are investigated in a laboratory fermenter under otherwise identical conditions. The composition of the medium for the preliminary and main stages is as follows:

1%	yeast extract
2%	peptone
	carbon source (in each case the same quantity based on the carbon content)
80 mg/l	adenine
80 mg/l	uracil
pH =	5.0

The main culture is inoculated with 2% inoculum and incubated for 48 hours.

The following results were obtained when ethanol and glucose were used

as carbon sources:

Carbon source	Hirudin concentration [%]	Optical density A_{578nm} [%]
2% glucose	100	100
1.54% ethanol	83	71

Example 3: Comparison of the recombinant preparation of hirudin in yeast by fermenting with or without glucose feeding

a) Fermentation with glucose feeding (fed-batch process)

1600 l of culture medium, which comprises one or more complex constituents, for example cornsteep, soyabean meal, yeast extract, etc., without glucose or other carbon sources, such as sucrose, being added, are sterilized at 121°C for 20 minutes in a fermenter having a total volume of 3600 l.

After it has been cooled down, the medium is inoculated with approx. 200 l of a preliminary culture which has been grown for approx. 12 hours (optical density A_{540nm} = 3.5 ± 0.5) and which still contains only very low residual quantities of glucose. The medium of the preliminary culture is equivalent to that of the main culture except that 1% glucose is added.

Immediately after the main stage has been inoculated, 2 kg of glucose are metered in per hour, in the form of a 20% aqueous solution and via a device for ensuring continuous sterilization, at a uniform rate until the end of the fermentation.

Aerobic conditions must be ensured during the fermentation; an oxygen partial pressure of $pO_2 \geq 20\%$ has to be maintained.

After 48 ± 2 hours, the fermentation is complete and is terminated by

adding 0.225 ± 0.025% benzalkonium chloride, e.g. 0.45 ± 0.05% ®Dodigen 226 (a 50% solution of a mixture of alkyldimethylbenzyl-ammonium chlorides in water).

Working-up then begins for isolating hirudin from the culture broth.

The final volume (medium + condensate + preliminary culture + metered-in glucose solution - water loss due to gassing) is 2300 ± 50 l.

b) Batch process

The batch process is equivalent to the fed-batch process described in a) with the following exceptions:

- Prior to inoculation, the medium contains 4% glucose in addition to the complex constituents.
- No further metering-in of glucose takes place.
- The final volume is only approx. 1800 ± 50 l.

c) Comparison of the results

Process	Hirudin concentration [%]	Hirudin quantity [%]
Batch process	100	100
Fed-batch process	228	291

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for producing a heterologous protein by fermentation of yeast using an ADH II promoter system, which comprises
   a) inoculating a main culture with a preliminary culture of a yeast strain which can express the heterologous protein wherein the expression of said gene is only under the control of the ADH II promoter;
   b) immediately after the inoculation supplying 0.7-1.4, preferably 0.9-1.3, and very particularly preferably 1.1, g of glucose/l of culture volume/h continuously at a constant rate;
   c) ensuring aerobic culture conditions;
   d) terminating the fermentation after approx. 2 days, and finally,
   e) isolating the heterologous protein from the fermentation culture.

2. The process as claimed in claim 1, wherein the media of the preliminary culture and the main culture are equivalent apart from an addition of 1% glucose to the preliminary culture medium.

3. The process as claimed in one of claims 1 and 2, wherein the 1% glucose which has been added to the preliminary culture medium has been almost completely consumed at the time of the inoculation.

4. The process as claimed in one of claims 1 to 3, wherein the aerobic culture conditions are ensured by means of an oxygen partial pressure of at least 20% saturation.

5. The process as claimed in one of claims 1 to 4, wherein said media contain one or more complex constituents, for example cornsteep, soyabean meal or yeast extract.

6. The process as claimed in one of claims 1 to 5, wherein said yeast strain is a Saccharomyces cerevisiae strain, preferably Y79.

AUSTRALIAN PATENT OFFICE

7. The process as claimed in one of claims 1 to 5, wherein said yeast strain is a Kluyveromyces lactis strain.

8. The process as claimed in one of claims 1 to 7, wherein the heterologous protein is a hirudin, a miniproinsulin, a leptin or a derivative thereof.

9. The process as claimed in claim 8, wherein the heterologous protein is a fusion protein which contains the amino acid sequence of a hirudin, a miniproinsulin, a leptin or derivatives thereof.

10. The process as claimed in any one of claims 1 to 9, wherein the yeast contains an expression vector containing a gene encoding the heterologous protein.

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