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(54) Title: PROCESS FOR THE PRODUCTION OF SECONDARY METABOLITES		
(57) Abstract The invention relates to an improved process for the production of a secondary metabolite comprising (i) fermentation of a microorganism capable of producing said secondary metabolite, and (ii) recovering said metabolite in substantially pure form. Said microorganism has been modified in a manner whereby the expression of one or more of the DNA sequences coding for (a) peptide(s), (a) protein(s) or (an) enzyme(s), involved in or interfering with the biosynthetic pathway of said secondary metabolite, is regulated differently from the regulation of said DNA sequence(s) in the original microorganism. Further contemplated is a process for production of said microorganism, a DNA construct, a vector or transformation vehicle, a microorganism capable of producing secondary metabolite and finally a secondary metabolite product.		

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Title: Process for the production of secondary metabolites

FIELD OF THE INVENTION

The present invention relates to processes for the production of secondary metabolites in high yields by use of modified microorganisms. The invention further relates to processes for producing the modified microorganisms, DNA constructs and vectors for use in such processes and the modified microorganisms. Lastly the invention relates to secondary metabolites produced by the first mentioned methods.

BACKGROUND OF THE INVENTION

The biochemical pathways of microorganisms can be classified as being part of either primary or secondary metabolism. The pathways of primary metabolism are involved in the catabolism of molecules for energy production or in the synthesis of the building blocks of the cells. Most of these processes are common for all microorganisms. The secondary metabolism is usually anabolic and leads to compounds with no obvious function for the cell.

Among secondary metabolites β -lactam antibiotics are a large family produced in nature by microorganisms. The most important class of β -lactam antibiotics both clinically and economically are the penicillins and the cephalosporins. Their biosynthesis occur via a complex pathway of enzymatic steps.

The unravelling of this pathway has been the subject of many studies during the last few decades. The first two steps in the biosynthetic pathways of the penicillin and the cephalosporin classes of β -lactam antibiotics are identical. Thereafter the biosynthetic pathways to the penicillins and cephalosporins diverge.

35 The β -lactam biosynthetic pathway

The pathway to the important penicillin species penicillin V is sketched below.

Aminoadipic acid + cysteine + valine → ACV-tripeptide → isopenicillin N → penicillin V

The first step is a condensation of L- α -aminoadipic acid (an intermediate in the lysine biosynthetic pathway in fungi or a degradation product of lysine in bacteria), L-cysteine and L-valine. In cephamycin-producing Actinomycetes, lysine (an amino acid belonging to the so-called aspartate family) is synthesized by the dihydrodipicolinate pathway, which does not include α -aminoadipic acid as an intermediate. In these organisms the precursor is formed by catabolism of lysine by the action of lysine-6-aminotransferase.

In the second step, ACV is oxidatively cyclized by removal of four hydrogen atoms to form the bicyclic penam nucleus (a β -lactam fused to a thiazolidine ring) of isopenicillin N which is present in all penicillins. From here the pathway diverges to penicillins in *Penicillium chrysogenum* and *Aspergillus nidulans* and to cephalosporins and cephamycins in various molds and Actinomycetes. Cephalosporins and cephamycins (7- α -methoxycephalosporins) contain the cephem bicyclic ring system (a β -lactam fused to a dihydrothiazine ring).

The enzymes catalyzing the β -lactam pathway

Formation of the ACV-tripeptide is carried out by the enzyme δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS).

The enzyme catalyzing the second step in the penicillin, cephalosporin and cephamycin biosynthesis is isopenicillin N synthase (IPNS or cyclase). IPNS is stimulated by ferrous ions and ascorbate, and requires a reduced environment. As the β -lactam ring is formed during cyclization, isopenicillin N (IPN) is the first compound produced in the pathway with antibiotic activity.

35

IPNS has been purified from a wide variety of β -lactam producing organisms including *Streptomyces clavuligerus*, *Streptomyces*

lactamdurans, *Penicillium chrysogenum* and *Cephalosporium acremonium*.

The final step in the penicillin biosynthesis is catalyzed by
5 acyl-CoA:6-aminopenicillanic acid transferase (AT), which has
been purified from organisms as e.g. *Penicillium chrysogenum*
and *Aspergillus nidulans*.

Most of the enzymes involved in the biosynthesis of β -lactams
10 have been characterized. The genes coding for ACVS, IPNS (cyc-
lase) and AT have been cloned and modified in different ways to
increase expression (Martin, J.F, J. Indust. Microorg., 9, p.
73-90, 1992).

15 The genes encoding the penicillin biosynthesis

The genes of penicillin biosynthesis in *P. chrysogenum* and *A. nidulans* are named respectively pcbAB, pcbC and penDE and are tightly clustered.

20 The pcbAB gene, encoding the ACV synthetase (ACVS) in *P. chrysogenum*, is an unusually large gene of about 12 kb without any introns (Smith et al., EMBO J., 9, p. 2743-2750, 1990, Diez et al., J. Biol. Chem., 265, p. 16358-16365, 1990). Transcriptional mapping showed the presence of a long transcript of about
25 11.5 kb that hybridized with several probes internally in the pcbAB gene. Further two small transcripts of 1.15 kb hybridized with the pcbC or the penDE gene (Martin, J. F., J. Indu. Micr., 9, p. 73-90, 1992).

30 The transcriptional initiation and termination region of the pcbAB gene has been completely sequenced. The pcbAB gene is linked to the pcbC and penDE genes and is transcribed in the opposite orientation to them.

35 Also the pcbC gene, encoding isopenicillin N synthase or cyc-lase of *P. chrysogenum* has been sequenced. The sequence does not contain any introns and the genes of *P. chrysogenum* (Barredo et al., Mol. Gen. Genet, 216, p. 91-98, 1989) are very

similar to those of *Streptomyces griseus*, *N. lactamdurans*, and other Actinomycetes and filamentous fungi.

The penDE gene encodes AT which is the last step of the penicillin biosynthesis (Barredo et al., Gene, 83, p. 291-300, 1989). The penDE gene of *A. nidulans* is very similar to the penDE gene of *P. chrysogenum* and contains three introns in similar positions.

10 Transcription of the genes of the penicillin biosynthesis

Several groups have reported transcription analysis of the upstream regions of the pcbC and pcbAB genes. In the fungi, the divergently transcribed genes are separated by about 1 kb . Smith et al. (Bio/Technology, 8, p. 237-240, 1990) used S1 mapping and primer extension to identify transcription initiation sites in the 5' region of the *C. acremonium* pcbC gene. Major and minor pairs of mRNA start sites were found on either side of a pyrimidine-rich block in the promoter region at positions -64 and -72, relative to the first base of the ATG initiation codon. A consensus TATA box was observed 68 bp upstream of the first major transcription start site. A similar motif was found at position -147 in the 5' region of the *A. nidulans* pcbC gene.

25 The sequence flanking the translation initiation codon matches the consensus fungal sequence. Barredo et al. (Mol. Gen. Genet., 216, p. 91-98, 1989) mapped the start site of *P. chrysogenum* pcbC mRNA by primer extension and showed that a single transcript was made that originated close to the structural gene, starting at position -11.

Similar studies by Kolar et al. (J. Biotechnol., 17, p. 67-80, 1991) with a penicillin production strain of *P. chrysogenum* revealed two major transcription initiation sites, at -131 and -132 as well as at - 397.

Primer extension studies of the *A. nidulans* pcbAB gene demonstrated a major mRNA start point at -230 bp. There was found no

recognizable core promoter sequences, a situation frequently encountered in fungal genes. As the pcbC and pcbAB genes may be regulated in a coordinate fashion, a search was made for potential regulatory elements, such as receptor sites for trans-
5 acting proteins, within the intergenic region separating the pcbAB and pcbC genes. A 53-bp region of dyad symmetry is located equidistant from the two genes, but no other extensive sequence identities were detected (McCabe et al., J. Biol. Chem., 266, p. 12646-54, 1991).

10

Analysis of pcbC mRNA during a *C. acremonium* seven-day fermentation showed a large accumulation of a 1.5-kb transcript between the second and the fourth day. This correlated with the appearance of products of the pathway after isopenicillin N
15 (Smith et al., Bio/Technology, 8, p. 237-40, 1990). The fact that mRNA levels decreased after the fifth day when antibiotics peaked was attributed to stability of the IPNS enzyme.

Regulation of the genes of the penicillin biosynthetic pathway

20 Little is known about the molecular mechanisms that modify the expression of the genes that regulate the penicillin biosynthesis, despite the fact that many studies show the biosynthetic pathway is subject to numerous metabolic controls.

25 Recent efforts in this direction have focused on characterizing the DNA regions controlling gene expression and analysing transcription events in terms of critical cell growth parameters that affect antibiotic formation.

30 ACV synthesis may be the rate-limiting step in biosynthesis of penicillins and cephalosporins and is known to be regulated by glucose in *Penicillium chrysogenum* and *Nocardia lactamdurans*, by phosphate in *Streptomyces clavuligerus* and by ammonium in *Streptomyces clavuligerus* and *Cephalosporium acremonium*. It is
35 also strongly affected by the oxygen transfer rate of the cultures.

Regulation of *pcbC* expression in *C. acremonium* occurs primarily at the transcriptional level. Similar studies of the *S. clavuligerus* *pcbC* gene show its expression to be under transcriptional control. When cultures of *S. clavuligerus* were grown in
5 rich or defined media, the amounts of *pcbC* mRNA correlated well with the IPNS enzyme activity and antibiotic production; in defined media, peak values of both occurred much earlier than in rich media (Y. Aharonowitz et al., Annu. Rev. Microbiol., 46, p. 461-95, 1992).

10

Analysis of mRNA levels of penicillin biosynthetic genes in *A. nidulans*, under conditions where the penicillin synthesis was repressed, showed no transcripts, suggesting common regulation of these genes at the transcriptional level (McCabe et al.,
15 EMBO J., 9, p. 279-87, 1990).

Penalva et al. (Genetics and Molecular Biology of industrial Microorganisms, Washington, DC, Am. Soc. Microbiol., p. 256-61, 1989; Gene, 89, p. 109-15, 1990) showed that in *A. nidulans* the
20 *pcbC* gene was transcribed only after arrest of cell growth and only then penicillin was detected in the fermentation broth.

A rather different picture was found in *P. chrysogenum*. Levels of *pcbC* mRNA and IPNS stayed about the same throughout the
25 fermentation, both in a wild-type strain and in a highly mutated overproducer strain (Kuck et al. Appl. Microbiol. Biotechnol., 31, p. 358-65, 1989). The latter exhibited 32- to 64-fold more mRNA than the wild-type strain.

30 Beatriz Pérez-Esterban et al. (Molecular Microbiology 9:4, p. 881-895, 1993) found that the IPNS promoter of the *A. nidulans* IPNS gene is mostly regulated by upstream negative control elements that act upon a high basal activity. Sequential deletion analysis of three negative cis-acting elements result in a
35 mutated promoter that is 40 times (sucrose broth) or 12 times (lactose broth) more active than the wild type. One of these cis-acting elements is involved in sucrose repression. Strikingly, it is located outside the non-transcribed 525 bp inter-

genic region and maps to the coding region of the divergently transcribed pcbAB gene. A 5'-deletion up to -56 (relative to the major transcription starting point (tsp)) showed that this region contain information to provide almost half of the maximal promoter activity and allows initiation of the transcription at the correct site. By using total-protein extract from mycelia grown under penicillin producing conditions a DNA-binding activity was detected which specifically binds to a promoter fragment located between -654 and -455 (relative to IPNS tsp). Deletions covering this region partially abolish IPNS promoter activity.

The interpretation of regulatory mechanisms in mutated, high- β -lactam-producing strains is complicated by possible chromosomal aberrations in the cluster of biosynthetic genes. For example, one *P. chrysogenum* overproducer strain had 8-10 copies of the pcbC gene (Smith et al., Mol. Gen. Genet., 216, p. 492-97, 1989) and another contained the pcbC and penDE genes in a DNA segment of at least 35 kb amplified 14-fold (Barredo et al., Curr. Genet., 16, p. 453-59, 1989). The significance of such findings is relevant for attempts to genetically manipulate high producer strains, either through introducing additional copies of β -lactam biosynthetic genes to overcome pathway blocks or by altering regulatory elements.

25

Amplification of the pcbC-penDE gene cluster of *P. chrysogenum* Wis 54-1255 led to as much as a 40% improvement in production yields (Veenstra et al. J. Biotechnol., 17, p. 81-90, 1991). Increased antibiotic yields were also reported in *A. nidulans* transformants containing multiple copies of pcbAB and pcbC genes (McCabe et al., J. Biotechnol., 17, p. 91-97, 1991).

Attempts to increase cephalosporin C yields in *C. acremonium* and penicillin in *P. chrysogenum* by inserting multiple copies of the pcbC gene were unsuccessful (Skatrud et al. Bio/Technology, 7, p. 477-86, 1989).

A similar result was obtained in terms of penicillin production in a wild-type strain of *A. nidulans* (Penalva et al., Genetics and Molecular Biology of industrial Microorganisms, Washington, DC, Am. Soc. Microbiol. p. 256-61, 1989).

5

Relevant patent documents

US patent no. 4,885,251 (Eli Lilly) describes a DNA sequence from *C. acremonium* encoding isopenicillin N synthase (IPNS). The IPNS encoding gene sequence was isolated from *C. acre-*
10 *monium*. The intact IPNS gene (pcbC) and associated promoter has been used to construct a vector that drives the expression of IPNS in *C. acremonium*. Further the IPNS promoter has been fused to a hygromycin phosphotransferase-encoding DNA sequence and placed onto *C. acremonium* expression vector.

15

US patent no. 4,892,819 (Eli Lilly) describes a DNA sequence, encoding isopenicillin N synthase (IPNS), comprising the IPNS encoding gene (pcbC) and its promoter from *Penicillium chrysogenum*. The DNA sequence can be placed in an expression vector
20 that function in *P. chrysogenum* and *C. acremonium*. This can be used to increase ultimate expression of a product encoded on a recombinant DNA vector.

EP 200,425 (Eli Lilly) discloses vectors encoding isopenicillin
25 N synthase (IPNS). The vectors permit high level expression of IPNS in *C. acremonium* and *E. coli*. The *Cephalosporium* vectors are useful for strain improvement, to increase efficiency and yield in fermentations for the production of penicillin and cephalosporin antibiotics. The vectors may also be modified to
30 give vectors for increasing the production yields and efficiency of *P. chrysogenum*, *Streptomyces clavuligerus* etc. in fermentations.

EP 260,762 (Gist-Brocades) provides a transformation method for
35 preparing *Penicillium* transformants. The DNA is preferably integrated into a host with stable expression of the structural gene(s) which is introduced. Particularly, complementation of auxotrophy is employed for selection.

EP 354,624 (Gist Brocades) describes a subtraction isolation method for identifying genes associated with the production of secondary metabolites in microorganisms. The method is exemplified with production of penicillin in *P. chrysogenum*.

5

EP 357,119 (Gist Brocades) discloses the clustered antibiotic biosynthetic genes encoding IPNS, AT and ACVS and are advantageously employed for improvement of production of the antibiotic in microorganisms and for the isolation of other genes
10 involved in the biosynthesis of the antibiotic. The invention is exemplified with improved production of penicillin in *P. chrysogenum*, with the isolation of another clustered biosynthetic gene(s) and with the expression of clustered penicillin biosynthetic genes in *Acremonium chrysogenum*.

15

EP 448,180 (Gist Brocades) describes a method for modulating production of secondary metabolites which includes modulating the number and/or the size of the organelles, preferably microbodies, in host organism. This is done by altering the
20 expression of a protein present in said organelles; and/or interfering with the cellular control mechanisms for maturation or fission of said organelles; and/or contacting the microorganism with agents capable of regulating the number and/or size of organelles; or modulating the cellular localization of at
25 least one protein, optionally derived from another microorganism, directly or indirectly involved in the production of said secondary metabolites by adding, deleting or altering one or more DNA sequences encoding one or more targeting signals in the gene(s) of one or more of said proteins.

30

Discussion of prior art

Prior art describes how to obtain high accumulated yields of secondary metabolites by increasing the number of copies of the structural gene(s) present in the fermentation microorganism
35 and/or modification of these genes.

However, increasing the number of copies of structural genes present in the fermentation microorganism does not necessarily

increase the yield of the secondary metabolites. Apart from incorporation of the extrachromosomal DNA into essential parts of the chromosomal DNA of the microorganism, this can also lead to an expression in a growth phase in which not all the enzymes
5 of the pathway are expressed or where the precursors for the pathway are unavailable. Furthermore, the lack of yield increase can be due to a complex set of interactions between the participants, in the biosynthetic pathways, e.g. precursors and intermediates. This may e.g. be the case when some of the
10 enzymes in the pathway are inhibited by intermediates or products from the pathway. Other examples are accumulation of (unstable) intermediates when bottlenecks arise due to limited enzyme levels or inhibited activity, which in turn may influence the recovery negatively, or when several pathways are com-
15 peting for the same intermediates.

Furthermore, it is often desirable to produce fermentable products by a continuous fermentation process as the process equipment is used more efficiently. However, this requires that
20 the genes involved in the biosynthesis of the metabolite must all be expressed by growing cells.

When the secondary metabolite is produced using immobilized cells, the cells are usually not growing. It is thus necessary
25 that the genes involved in the biosynthesis of the metabolite are expressed under no-growth conditions.

More specifically, in the fermentation of e.g. penicillin V, if the side-chain precursor is added to the fermentation continu-
30 ously during the production, the penicillin V slowly starts to accumulate in the broth after a lag phase. The volumetric productivity does not reach a reasonable level until the culture is several hours old. If no side chain precursor is added to the fermentation a mixture of isopenicillin N, 6-aminopenicil-
35 lanic acid and various "natural" hydrophobic penicillins will accumulate. The presence of these β -lactams represents a waste of substrate and may interfere with the following recovery of the desired penicillin.

SUMMARY OF THE INVENTION

The object of the invention is to overcome some of the above mentioned problems by providing an improved process for the production of a secondary metabolite comprising i) fermentation
5 of a microorganism capable of producing said secondary metabolite, and ii) recovering said metabolite in substantially pure form.

Said microorganism has been modified in a manner whereby the
10 expression of one or several of the DNA sequences coding for (a) peptide(s), (a) protein(s) or (an) enzyme(s), involved in or interfering with the biosynthetic pathway of said secondary metabolite, is regulated differently from the regulation of said DNA sequence(s) in the original microorganism.

15

Said expression may be initiated at either an earlier or a later fermentation stage in comparison to the original microorganism.

20 The expression level of said peptide(s), protein(s) or enzyme(s) may be increased at an earlier or later fermentation stage in comparison to fermentation of the original microorganism. The expression may be maintained throughout the fermentation.

25

According to a preferred embodiment the modification of the microorganism is accomplished by substitution of the promoter(s) region(s) regulating the expression of said DNA sequence(s).

30

Another object of the invention is to provide a DNA construct comprising a gene encoding the peptide(s), protein(s) or enzyme(s) of interest being connected in a regulatory manner to regulatory element(s)/promoters, which will lead to a regulation
35 tion that differs from the regulation of the original microorganism.

A specific embodiment of this aspect of the invention relates to a DNA construct comprising the IPNS structural gene (pcbC) and the terminator, which is expressed under control of a penDE gene promoter (ATp).

5

A still further object of the invention is to provide a vector or transformation vehicle comprising such a DNA construct.

Also the invention relates to a process for the production of
10 a microorganism capable of producing a secondary metabolite, which microorganism has been modified in a manner whereby the expression of one or several of the DNA sequences coding for (a) peptide(s), (a) protein(s) or (an) enzyme(s), involved in or interfering with the biosynthetic pathway of said secondary
15 metabolite, is regulated differently from the regulation of said DNA sequence(s) in the original microorganism.

In a specific embodiment the invention relates to such a microorganism capable of producing clavulanic acid, indole
20 dihydrodiol and antibiotics, especially penicillin,.

Finally the invention relates to a secondary metabolite produced by a method according to the first aspect of the invention, especially an antibiotic, clavulanic acid or indole
25 dihydrodiol.

Preferred antibiotics are penicillins, such as penicillin G and penicillin V.

30 BRIEF DESCRIPTION OF THE DRAWING

The invention will be described in further details in the following parts of the specification with reference to the examples and figures.

35 Figure 1 shows the construction of the pME1301 plasmid, wherein (*) is the ligation site of the three fragment.

Figure 2 shows the restriction map of *P. chrysogenum* DNA region comprising the pcbAB, pcbC and penDE genes.

Figure 3 shows penicillin V yield and the activity levels of two penicillin biosynthetic enzymes, cyclase and acyl-transferase, in B10 during a fermentation in shake flasks. The activity is relative to the maximal activity found during the 5 fermentation.

Figure 4 shows the penicillin yields in shake flasks after 3 and 5 days of fermentation for B10 and two ATp IPNS transformants (130 and 137).

Figure 5 shows the relative penicillin yield for a 1 liter 10 batch fermentation of B10 and transformants 130 and 137. The curve for B10 is an average of 5 fermentations while the curves for the two transformants each represents the average yields from two fermentations.

15 DETAILED DESCRIPTION OF THE INVENTION

The invention relates to an improved process for the production of a secondary metabolite comprising i) fermentation of a microorganism capable of producing said secondary metabolite, 20 and ii) recovering said metabolite in substantially pure form.

Said microorganism has been modified in a manner whereby the expression of one or more of the DNA sequences coding for (a) peptide(s), (a) protein(s) or (an) enzyme(s), involved in or 25 interfering with the biosynthetic pathway of said secondary metabolite, is regulated differently from the regulation of said DNA sequence(s) in the original microorganism.

In an embodiment of the process according to the invention said 30 expression is initiated at a different fermentation stage in comparison to the original microorganism. If the expression is initiated at the same time the expression is maintained throughout the fermentation.

35 The fermentation stage includes, e.g., the lag, growth and stationary phases.

DNA sequences coding for peptide(s), protein(s) or enzyme(s) interfering with the biosynthetic pathway of said secondary metabolite may e.g. be competing (a) peptide(s), protein(s) or enzyme(s) involved with competing pathways.

5

In another embodiment the expression level of said peptide(s), protein(s) or enzyme(s) is increased at an earlier or later fermentation stage in comparison to fermentation of the original microorganism. The expression may be maintained
10 throughout the fermentation.

An increased expression level of the peptide(s), protein(s) or enzyme(s) in question is defined as an increased activity level.

15

The alteration of the regulation of the expression of the peptide(s), protein(s) or enzyme(s) may be obtained by genetic modification of one or more of the DNA sequence(s) of said original microorganism. Suitable modifications are e.g.
20 substitution of the promoter and mutations at specific site(s) of the DNA sequence(s) responsible for regulating the initiation and expression of the gene(s). An example of a possible mutation is deletion and addition of one or more bases, using well-known procedures for site-directed or random mutagenesis,
25 e.g. through radiation or chemical treatment.

Further contemplated according to the invention is modification by substitution of the DNA sequence(s) or at least a region in the DNA sequence(s) regulating the initiation and expression of
30 the gene(s).

In a preferred embodiment of the invention said modification is accomplished by substitution of the promoter(s) region(s) regulating the expression of said DNA sequence(s).

35

The promoter may be any DNA sequence which regulates the expression of said DNA sequences differently from the promoter in the original microorganism.

The above mentioned principles of the process of the invention may be used for the production of any industrially important secondary metabolite where a coordinated expression of one or more peptides, proteins or enzymes is advantageous.

5

Examples of such relevant secondary metabolites include penicillins, cephalosporins, cephamycins, mono-bactams, chloramphenicol, erythromycin, streptomycin, clavulanic acid, nocardicins, and indole dihydrodiol.

10

For instance, a coordinated expression of several peptides, proteins or enzymes is advantageous in the production of indole dihydrodiol (for indigo-dyes). Indole dihydrodiol can be produced in *E. coli* after the introduction of a *Pseudomonas* 15 *putida* naphtalene dioxygenase (see Murduoch et al., (1993), Bio/Technology 11, 381).

The precursor for the dioxygenase in indigo synthesis is indole which is an intermediate in the biosynthesis of tryptophane.

20 Indole is usually only present in the cell in low concentrations, but by genetic engineering of the enzyme producing it (i.e. tryptophane synthetase) higher indole levels can be obtained.

25 In order to stabilize the naphtalene dioxygenase the simultaneous expression of a peptide, ferredoxin, is necessary. By using the same promoters for naphtalene dioxygenase and ferredoxin the stabilization can be obtained.

30 Further, the use of the same promoter for the modified tryptophane synthetase will lead to a simultaneous production of the substrate, indole.

By limiting the expression of the enzymes to a production 35 phase, the metabolism of the cell will not be loaded with the extra burden required for the production of these peptides, proteins or enzymes in e.g. the growth phase where the buildup of an active cell mass is most important. Another advantage by

limiting the time where the genes are expressed is that the likelihood of genes reverting is limited.

The above points to the general applicability of the principle of the invention to metabolically engineered amino acid overproducing cells where it is important that all genes involved in the biosynthesis are expressed coordinately.

Other examples are the production of phenylalanine (Ikeda, M. and Katsumata, R. (1992) Appl. Envir. Microbiol. 58, 781) or amino acids of the aspartate family (Jetten, M.S.M., and Sinskey, A.J. (1995) Crit. Rev. Biotech. 15, 73).

In the following the production of the antibiotic penicillin will be used as another specific example. It is to be emphasized that penicillin production should only be regarded as an example for illustrating the general principle of the invention.

The penicillin to be produced may for instance be penicillin G or penicillin V.

By controlling the expression of the enzymes in the biosynthetic pathway an earlier onset of the penicillin production can be obtained.

The part of the DNA sequence encoding penicillin to be controlled differently according to the invention may be the region coding for the isopenicillin N synthase (cyclase or IPNS) enzyme.

More specifically said DNA sequence may be modified so that the IPNS promoter is substituted by the promoter regulating the expression of the acyl-CoA:6-amino penicillanic acid transferase enzyme (AT) from *P. chrysogenum*.

AT is mainly produced early in the fermentation of penicillins while the cyclase peaks when growth decreases. Thus, the combi-

nation of the AT-promoter with the cyclase gene will give an earlier expression of this enzyme and an earlier penicillin production.

5 In a specific example the original microorganism is *Penicillium chrysogenum* used for producing penicillin. The AT is expressed at a high level in early stage of the fermentation. The IPNS activity increases after a considerable period of fermentation. In order to reduce this lag phase and to induce the penicillin
10 biosynthesis earlier in fermentation, the fermentation strain was transformed with a plasmid comprising the IPNS gene with the AT promoter (ATp IPNS gene).

The exchange of the promoter(s) can result in a coordinated
15 expression of all biosynthetic enzymes for a given metabolite. This leads to a production without accumulation of intermediates in certain phases of the fermentation with the risk of toxic effects or degradation.

20 A synchronization of the expression of the biosynthetic enzymes can lead to a faster production without or with reduced accumulation of intermediates, which can give purification problems or inhibition of the biosynthesis. It also reduces the degradation of already present β -lactam.

25

It is advantageous to use the concept of this invention in connection with immobilized cells. Immobilized cells are usually not growing and it is thus necessary to have expression of the biosynthetic genes under no-growth conditions. Some biosyn-
30 thetic enzymes are primarily produced by growing cells and it is therefore advantageous to introduce a promoter giving increased levels of biosynthetic enzymes in stationary growth phase.

35 According to the present invention it is possible to select a production phase which is more suitable to the needs of the production. An earlier start of the biosynthesis of the secondary metabolite during the production will lead to a

better utilization of the equipment and in some cases to a higher accumulated yield. Alternatively, a delayed production phase may be desirable in order to e.g. build up a certain biomass before the cells start the biosynthesis of the desired metabolite. This may especially be advantageous if the metabolite is unstable at the conditions used during the cell growth phase or if the metabolite itself or one of its precursors is able to inhibit cell growth. A delayed production phase may then result in a more concentrated production phase, a reduced loss of a labile product, which in turn, leads to the formation of less byproducts, which can be of large significance for the recovery of the metabolite.

Another argument for a period with a concentrated production phase is labile precursors or products. This can either be due to chemical instability or due to the utilization for other metabolic purposes.

An alternative is a constitutive production of a metabolite, i.e. production in all growth phases. In a continuous fermentation it is necessary that all the enzymes are expressed under conditions where at least some growth occur. Therefore regulation factors that allow expression of all biosynthetic enzymes are usually advantageous under these circumstances.

25

Alternatively a maintenance of expression during growth is necessary in a continuous fermentation process, since it is necessary that all the enzymes are expressed under conditions where growth occurs.

30

Whether an early or late expression of the biosynthetic enzymes is preferable is very dependent on the specific case. Factors, which may influence the choice, are toxic effects, stability of precursors, intermediates or product, mode of production (e.g. immobilized cells), ease of recovery, and economy.

Another application of the principle is for the regulation of competing metabolic pathways. In some circumstances it will be

advantageous to shut down or minimize this competition during the production phase of a metabolite.

This is the case if the competing pathways is draining the
5 primary metabolites which are used for the biosynthesis of the desired product or if the product could itself be metabolized. Further if the primary pathways produce known repressors of the biosynthesis of the desired product.

10 Another object of the invention is to provide a process for the production of a microorganism capable of producing a secondary metabolite. An original microorganism is modified in a manner whereby the expression of one or more of the DNA sequences coding for (a) peptide(s), (a) protein(s) or (an) enzyme(s),
15 involved in or interfering with the biosynthetic pathway of said secondary metabolite, is regulated differently from the regulation of said DNA sequence in the original microorganism.

In an embodiment of the invention said original microorganism
20 is modified in a manner whereby said expression is initiated at a different fermentation stage in comparison to the original microorganism. If the expression is initiated at the same time the expression is maintained throughout the fermentation.

25 It is also contemplated to increase the expression level of said peptide(s), protein(s) or enzyme(s) at an earlier or a later fermentation stage in comparison to fermentation of the original microorganism. The expression may be maintained throughout the fermentation.

30

In a preferred embodiment of the invention said original microorganism is modified by substitution of the promoter(s) region(s) regulating the expression of said DNA sequence(s).

35 According to the invention said DNA sequence(s), e.g. comprising the promoter(s) or structural gene(s), may be isolated by well-known methods. Thus, the DNA sequence may, for instance, be isolated by establishing a cDNA or genomic library from an

organism expected to harbour the sequence, e.g. a cell as described below, and screening for positive clones by conventional procedures. Examples of such procedures are hybridization to oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

A preferred method of isolating a DNA sequence from a cDNA or genomic library is by use of polymerase chain reaction (PCR) using degenerate oligonucleotide probes prepared on the basis of the DNA sequence in question. For instance, the PCR may be carried out using the techniques described in US Patent No. 4,683,202 or by R.K. Saiki et al., Science 239, p. 487-491, 1988.

Alternatively, the DNA sequence may be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beaucage and Caruthers, 1981, or the method described by Matthes et al., 1984. According to the phosphoramidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA molecule, in accordance with standard techniques.

Preferably said original microorganism belongs to a genus from the group comprising *Penicillium*, *Cephalosporium*, *Aspergillus*, *Nocardia*, *Streptomyces*, *Bacillus*, *Pseudomonas*, *Cercospora*, *Micromonospora*, other *Eubacteria*, other *Actinomycetes* or filamentous fungi, capable of producing industrially important secondary metabolites selected from the group comprising penicillins, cephalosporins, cephamycins, mono-bactams,

chloramphenicol, erythromycin, streptomycin, clavulanic acid, nocardicins and indole dihydrodiol.

In a preferred embodiment said original microorganism belongs
5 to a species from the group comprising *Penicillium chrysogenum*,
Acremonium chrysogenum, *Aspergillus nidulans*, *Nocardia lactam-*
durans, *Streptomyces clavuligerus* and *Pseudomonas putida*.

In another preferred embodiment said original microorganism is
10 a microorganism capable of producing penicillin, especially
penicillin G or penicillin V.

In still an embodiment of the invention said DNA sequence
comprises the DNA sequence coding for the isopenicillin N
15 synthase (cyclase or IPNS) enzyme.

In a specific embodiment the IPNS promoter is substituted by
the promoter (ATp) regulating the expression of the acyl-CoA:6-
amino penicillanic acid transferase enzyme (AT).

20

A further object of the invention is to provide a DNA construct
comprising (a) gene(s), which is(are) expressed under the con-
trol of a promoter different from the original promoter.

25 In the DNA construct, a DNA sequence, corresponding to the
gene, should be operably connected to a suitable promoter se-
quence or other expression regulating factors. The promoter may
be any DNA sequence which initiates expression of the
peptide(s), protein(s) or enzyme(s) at a growth stage different
30 from that of the original microorganism, and may be derived
from genes encoding proteins either homologous or heterologous
to the host cell.

The DNA construct may also comprise a suitable terminator
35 operably connected to the DNA sequence.

In an embodiment of the invention said DNA construct comprises
genes encoding enzymes involved in the secondary metabolism,

preferably some of the structural gene(s) encoding proteins for the β -lactam antibiotic biosynthesis, such as the structural gene(s) encoding enzymes for the penicillin biosynthesis.

- 5 In a preferred embodiment said DNA construct comprises structural gene(s) encoding the isopenicillin N synthase (cyclase or IPNS) and a promoter of genes involved in β -lactam antibiotic biosynthesis.
- 10 In still another preferred embodiment the promoter is a promoter of the genes involved in penicillin biosynthesis, such as the promoter of the acyl-CoA:6-amino penicillanic acid transferase (AT) gene (penDE).
- 15 In a specific embodiment of the invention said DNA sequence comprises the IPNS structural gene (pcbC) and the terminator expressed under control of the AT gene (penDE) promoter (ATp).

Alternatively the DNA sequence comprises a promoter of any of
20 the genes e.g. in the primary metabolism.

A further object of the invention is to provide a vector or transformation vehicle comprising a DNA construct. Said DNA construct is preferably one of the above mentioned DNA con-
25 structs.

The DNA construct may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmid
30 pBR322, PUC19, pACYC177, pUB110, pE194, pAMB1, pJC720 and pIJ702.

The DNA construct and/or vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect
35 in the host cell, such as the dal genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as pleomycin, ampicillin, kanamycin, chloramphenicol or tetra-

cyclin resistance, or positive selective markers, such as hygromycin B, AndS, sC, PyrG, ArgB, TrpC or NiaD.

To direct the enzyme to the desired location within the host cell or into the fermentation media, a targeting signal or a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence), respectively, may be provided in the recombinant vector. The targeting signals or secretory signal sequence are joined to the DNA sequence encoding the enzyme in the correct reading frame. Secretory sequences are commonly positioned 5' to the DNA sequence encoding the enzyme, whereas the targeting signal sequences are commonly positioned 3' to the DNA sequence. The targeting signal or secretory signal sequences may be that normally associated with the enzyme or may be from a gene encoding another protein having the desired signal sequence.

Intracellular expression is advantageous according to the present invention.

20

The procedures used to construct the DNA construct of the invention comprising ligating the DNA sequence(s) encoding the structural gene(s) in question, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., supra, 1989)).

In an specific embodiment the vector or transformation vehicle is pUC19 comprising an operably linked phleomycin resistant expression unit.

Also an object of the invention is to provide a microorganism capable of producing a secondary metabolite. The microorganism has been modified in a manner whereby the expression of one or more of the DNA sequences coding for (a) peptide(s), (a) protein(s) or (an) enzyme(s), involved in or interfering with the biosynthetic pathway of said secondary metabolite, is

regulated differently from the regulation of the DNA sequence(s) in the original microorganism.

Preferably the microorganism is produced by the above mentioned process and modified by transformation of a vector or DNA construct of the previous mentioned type.

The microorganism may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome, although the DNA construct may also exist as an extrachromosomal entity. However, the integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the microorganism. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous recombination. Alternatively, the microorganism may be transformed with an expression vector as described below in connection with the different types of host cells.

The microorganism of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Preferably the microorganism belongs to a genus from the group comprising *Penicillium*, *Cephalosporium*, *Aspergillus*, *Nocardia*, *Streptomyces*, *Escherichia*, *Bacillus*, *Pseudomonas*, *Cercospora*, *Micromonospora*, other *Eubacteria*, other *Actinomycetes* or filamentous fungi and is further capable of producing an industrial important secondary metabolite. These are preferably from the group penicillins, cephalosporins, cephamycins, monobactams, chloramphenicol, erythromycin, streptomycin, clavulanic acid, nocardicins and indole dihydrodiol.

The transformation of bacteria, such as *E. coli*, may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The filamentous fungus may belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se.

In a preferred embodiment the microorganism belongs to a species from the group comprising *Penicillium chrysogenum*, *Acremonium chrysogenum*, *Aspergillus nidulans*, *Nocardia lactam-*
10 *durans*, *Streptomyces clavuligerus*, *Pseudomonas putida* or *E. coli*.

The medium used to cultivate the microorganisms may be any conventional medium suitable for growing the host cell in
15 question. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

A final object of the invention is to provide a secondary
20 metabolite produced by the above mentioned method.

Preferably the secondary metabolite is an industrially important secondary metabolite selected from the groups of penicillins, cephalosporins, cephamycins, mono-bactams,
25 chloramphenicol, erythromycin, streptomycin, clavulanic acid and nocardicins, especially penicillin, such as penicillin G or penicillin V and pigments, in particular indigo-dyes.

MATERIALS AND METHODS

30 Strains:

B10: *P. chrysogenum* strain (available from Panlabs, 11804 North Creek Parkway South, Bothell WA 98011-8805, USA)

Strain 130: B10 transformed with pME1301 comprising the ATP IPNS gene

35 Strain 137: B10 transformed with pME1301 comprising the ATP IPNS gene

Clones:

Lambda KNK31 clone: Clone containing the penicillin gene cluster, respectively the pcbAB, pcbC and penDE genes.

Vector:

- 5 pUC19: 2.6kb Asp719-SalI fragment
pBR322: 3.9kb NheI-SalI fragment

Transformation selective marker:

- E. coli* Tn5 phleomycin resistant gene expressed by *Aspergillus*
10 *oryzae* TPI (triose phosphate isomerase) promoter and
Aspergillus niger AMG (Amyloglucosidase) terminator.

Plasmid:

- pME1243: 2.3kb EcoRI-SalI fragment penDE promoter -
15 pcbC gene - pcbC terminator in pUC19.
pME1205: Subclone of pcbC and penDE genes from lambda
KNK31 in pBR322.
pME1301: 7.6kb Plasmid containing penDE promoter - pcbC
gene - pcbC terminator (2.3kb), TPI promoter (0.9kb),
20 Tn5 phleomycin resistant gene (1.1kb), AMG terminator
(0.7kb) and pUC19 (2.6kb).

Materials:

- 25 LCS slant agar: (J. Lein, 1986, "The Panlabs penicillin strain
improvement program" in Vanek Z, Hostálek Z (eds.)
Overproduction of microbial metabolites, strain improvement and
process control strategies. Butterworth, Boston, p. 105-139).
P-1 seed medium: (J. Lein, 1986)
30 P-2 fermentation medium: (J. Lein, 1986, lard oil replaced by
olive oil))
Tween-80®: (Merck Art. 822187)
LCS medium: Lactose monohydrate 1.5%, corn steep liquor 0.5%
peptone 0.5%, NaCl 0.4%, MgSO₄·7H₂O 0.05%, KH₂PO₄ 0.06%,
35 FeCl₃·6H₂O 0.0005%, CuSO₄·5H₂O 0.002%, pH 4.8
LCS agar: LCS medium, 2% agar.
Myra cloth (Calbiochem)

Solutions for protoplast formation:

BSA: Bovine serum albumin (Sigma A7638)

Solution A: 5 ml contains 1.2 M MgSO_4 , 10 mM NaH_2PO_4 , pH 5.8, 150 mg Novozym234 (batch #1199), 100 μl chitinase (Sigma, 4U/ml).

5 Solution B: 0.6 M sorbitol, 100 mM Tris-HCl, pH 7.0

Solution C: 1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl_2

Solution D: 60% PEG4000(BDH#29576), 10 mM Tris-HCl, pH 7.5, 10 mM CaCl_2 .

10 Methods:

DNA was prepared by CsCl density gradient (Maniatis et al., 1982, Molecular cloning, A laboratory manual, Cold Spring Harbor Laboratory, New York).

15 Cloning of the penicillin gene cluster from *P. chrysogenum*.

Genomic DNA from *P. chrysogenum* was isolated according to the procedure of Schwarz-Sommer et al., EMBO J., 3, p. 1021-1028, 1984) and partially digested with Sau3A. Fragments 15-23 kb in size were isolated and ligated to lambda EMBL3 BamHI arms

20 (Promega).

By screening of the genomic library lambda EMBL3 with plaque hybridization method (Maniatis et al., 1982, supra) a clone was found, which hybridised with probes specific for pcbAB, pcbC

25 and penDE, i.e.:

(1) an approx. 400 bp PstI/BamHI fragments containing the pcbAB terminator,

(2) an approx. 1 kb NcoI fragment containing the pcbC coding region, and

30 (3) approx. 400 bp Bsu36I/SalI fragment containing the penDE terminator.

Lambda KNK31 DNA hybridised with all the above mentioned probes (see figure 2).

35 Restriction enzyme sites in lambda KNK31 DNA:

Restriction of lambda KNK31 with SalI cuts away the two lambda KNK31 arms (9 and 20 kb, respectively) from the insert, enabling a size estimation of the insert. There are no EcoRI

sites in the lambda arms, and the BamHI site used for cloning will statistically be maintained in 25% of the cases when ligated to Sau3A partials. The lambda KNK31 has an 18.6 kb insert and give rise to 6 SalI fragments, 4 EcoRI fragments and 5 3 BamHI fragments.

Transformation

Penicillium chrysogenum production strain B10 was cultured in 2 flasks of 100 ml LCS medium for 36 hrs. at 26°C. Mycelia was 10 collected on Myra cloth and washed thoroughly with 500 ml 0.6M MgSO₄, Mycelia was then transferred into a plastic flask and suspended in 5 ml Solution A and placed on ice for 5 min. 750 µl BSA (12 mg/ml) was added to the mycelial suspension and further incubated at 30°C for 1 to 2 hours with gentle shaking. 15 Protoplast formation was controlled with light microscope.

Protoplasts were collected on Myra-cloth and layered carefully over 5 ml of Solution B. After centrifugation slowly up to 2000 rpm for 15 min., protoplasts, which localized at the interphase 20 between Solution B and Solution A, were taken by pipeting and diluted by adding 2 vol. of Solution C and again centrifuged at 2500 rpm for 5 min. Protoplast pellet was washed twice with Solution C and isolated protoplasts were diluted by adding Solution C to get $1-2 \times 10^7$ cells/ml. 100 µl of protoplasts was 25 used for a transformation.

10 µg DNA prepared by CsCl density gradient was added to the protoplasts and left at room temperature for 20 min. Then 200 µl of Solution D were added and the mixture was left at room 30 temperature for 20 min. 3 vol. of 1.2 M sorbitol were then added and protoplast-DNA aggregate was centrifuged at 2500 rpm for 10 min. The pellet was then resuspended by adding 300µl 1.2 M sorbitol and plated on 3 selective plates (100 µl each) containing 50 µg/ml phleomycin and 1.2 M Sorbitol in the LCS 35 agar. Plates were incubated at 26°C until transformants appeared.

Fermentation of *P. chrysogenum*:

Lyophilized spores of *P. chrysogenum* (Panlabs B10 strain), were inoculated on 10 ml LCS slant agar after suspension in distilled water containing 0.1% (v/v) Tween-80®. After incubation at 25°C for 10 days, the spores from the slant surface were suspended in 10 ml of 0.1% Tween-80® and 5 ml were used to inoculate 50 ml P-1 Seed Medium in 300 ml erlenmeyer flasks. The seed culture were incubated at 25°C on a rotary shaker at 290 rpm. After 48 hours, 2 ml aliquots of seed culture were used for inoculation of the P-2 fermentation medium, 35 ml in 300 ml erlenmeyer flasks. The cultures were incubated at 25°C at 290 rpm until harvested.

Determination of penicillin V in supernatant from fermentation broth.

15 Firstly, 1 ml of ice cold ethanol was added to 1 ml supernatant, after mixing the proteins were allowed to precipitate for 5 min. on ice bath.

Secondly the samples were centrifuged for 5 min. at 3000 rpm (1550 g), 4°C. The supernatant was diluted with 50 mM Tris/HCl, 20 pH 7.2.

When analysing B10, samples from day 1-2 were normally diluted 200 times and samples from day 3-7 were diluted 2000 times (incl. the 1 fold dilution with ethanol)

25

The samples were analyzed by HPLC under the following conditions:

Eluent: 100 ml 25 mM Na-phosphate buffer, pH 7.0

180 ml acetonitrile

30 720 ml Milli-Q water

mixed and degassed by He

Flow: 1.5 ml/min

Detection: UV 210 nm

Column temp. 35°C

35 Column: Supelcosil LC-18-DB (25x4.6 mm) with Supelguard column

Runtime: 12 min.

Retention time (penicillin V): approx. 8.3 min.

Standard: 50 μ M Penicillin V (potassium salt)

Preparation of cell extracts from *P. chrysogenum* by sonication

Shake flasks were placed on ice bath until harvest. The cells
5 were harvested as quickly as possible by filtration on Büchner-
funnel coated with one layer Myra cloth (from Calbiochem). Then
the cells were washed with at least 2 volumes ice cold 0.9%
(W/V) NaCl on the funnel. 2 grams of the cells were weighed
into ice cold glass centrifugation tubes, followed by addition
10 of 4 ml 50 mM Tris/HCl, pH 7.2, 5 mM dithiothreitol, 1 mM EDTA
and 0.5 mM phenylmethylsulfonyl fluoride (PMSF).

Sonication took place on ice bath for 2 min. (50% duty cycle)
followed by centrifugation for 20 min. at 10,000 g. The
15 supernatant was desalted on PD10 column (G25) equilibrated with
the same buffer as used for resuspension of the cells, however
the PMSF content were reduced to 50 μ g/l. For the desalting 2
ml sample was applied on the column followed by 0.5 ml buffer
and eluted with another 2.5 ml buffer. The eluate containing
20 the extract was diluted 10 times with 50 mM Tris/HCl, pH 7.2,
5 mM dithiothreitol, 1 mM EDTA, no PMSF. Assay as described in
F-9200088 (Available on request from Novo Nordisk A/S).

EXAMPLES

25

Example 1

The entire penicillin biosynthetic gene cluster including the
ACV synthetase gene (pcbAB) was cloned from *P. chrysogenum* B10
30 in lambda EMBL3.

A phage (lambda KNK31) was found to contain the entire gene
cluster.

35 DNA was isolated (Maniatis et al., supra, 1982) from the lambda
KNK31 clone and the insert was mapped by restriction enzyme
SalI, EcoRI and BamHI. The restriction enzyme map is displayed
in Figure 2 and below in Table 1.

Table 1

lambd a KNK	Insert kb	SalI frag- ments kb	EcoRI fragments kb	BamHI fragments kb
5 31	18.6	1.6, 2.6, 6.0, 2.2, 5.1, 1.1	2.5, 7.9, 0.6, 4.3	4.7, 2.7, 2.3

Example 2DNA construct

10 Clone lambda KNK31 was digested with XbaI and BglII isolating a 1427 bp fragment comprising the pcbC gene and the penDE terminator (see Figure 2).

A 874 bp fragment comprising the penDE promoter was made by PCR
15 (PCR Protocols: A Guide to Methods and Applications, Ed. M.A. Innis, D.H. et al. Academic Press, Inc, 1990)), using lambda KNK31 as a template, and the following primers. The primer tail #1069M contains XbaI site instead of the XmnI site at the original AT promoter.

20

Primer #1069M:

XbaI

5'- CTAG TCTAGA GCGGGTCGGAAGATGGGTAAAC - 3'

25 Primer #1070M:

SacI

5'- TTCGGCAC GAGCTC TCCTTG - 3'

The PCR fragment was digested with SacI and XbaI restriction
30 enzymes and the 874 bp fragment was isolated by 0.7% agarose gel electrophoresis. This was ligated to the above mentioned XbaI-BglII fragment of 1427 bp, which contains pcbC structural gene and terminator and 2.6 kb pUC19 vector which was cut with SacI and BamHI enzymes (pME1243). The former fragments was
35 isolated from pME1205 (a subclone of pcbC and penDE genes from lambda clone, KNK31). From pME1243, 2.3 kb EcoRI-SalI fragment,

(penDE promoter, pcbC structural gene and pcbC terminator) (ATp IPNS) was taken out and ligated with 2.7 kb Asp718-EcoRI fragment of *E.coli* Tn5 phleomycin resistant gene expression unit (*Aspergillus oryzae* TPI promoter and *Aspergillus niger* AMG 5 terminator) and with pUC19 vector (2.6 Asp718-SalI fragment) This resulted in the pME1301 plasmid (see Figure 1).

B10 was transformed with pME1301 comprising the ATp IPNS gene. This lead to B10 transformants strain 130 and 137.

10

Example 3

Biosynthetic enzymes during a fermentation.

The appearance of cyclase and acyltransferase during a shake
15 flask fermentation of *Penicillium chrysogenum* B10 is shown in
Figure 3. The activities at 0 hours are the activities in the
preculture immediately before transfer into the production
medium. From the Figure 3 it is clear that acyltransferase is
primarily expressed early in the fermentation while cyclase
20 activity reaches a maximum late in the fermentation. Thus the
activities of the two biosynthetic enzymes are uncoordinated.
It appears from the figure that the production of penicillin
closely follows the curve for cyclase activity.

25 Example 4

Test of ATp IPNS transformants (130 and 137)

The penicillin production and cyclase activity in the two
transformants (130 and 137) produced according to example 2
30 were tested in shake flasks and compared to the host strain,
B10 and the results are given in Table 2, Table 3 and Figure 4.

It appears that the penicillin production is started earlier in
the two transformants compared to B10 and that the accumulated
35 penicillin V production in five days old cultures is increased
as well. This corresponds well with the increased cyclase
activity both the early and late stages of the fermentation.

In Table 2 the relative cyclase activity and penicillin V yield in 5 day shake flask cultures of ATP IPNS transformants (strain 130 and 137) are displayed. Yields are relative to B10.

5 Table 2

Strain	n*	Penicillin V yield (% of B10)	IPNS activity (% of B10)
B10	61	100 ± 10	100
130	10	115 ± 9	196
137	10	123 ± 9	141

10 : n is the number of shake flask fermentations of each strain.

By replacing the promoter for cyclase with the promoter from acyltransferase an increased activity of cyclase was obtained in the early part of the fermentation. Furthermore, the 15 elevated level of cyclase activity were maintained throughout the fermentation as is clear from Table 3.

In Table 3 is displayed the relative cyclase activity in 2 and 5 day old shake flask cultures of B10 and two ATP IPNS 20 transformants.

Table 3

Strain	Day 2	Day 5
B10	100 (n=5)*	100 (n=61)
130	155 (n=5)	196 (n=10)
137	148 (n=5)	141 (n=10)

25

*: n is the number of shake flasks fermentations of each transformant.

The two ATP IPNS transformants (130 and 137) were tested in 1 30 liter fermenters using the same medium as used in the shake flasks, and the relative accumulated penicillin V yields are given in Figure 5. From this figure it is evident that by replacing the promoter for cyclase with the promoter for

acyltransferase, the penicillin lag phase is reduced and a higher total yield of penicillin was obtained.

It is thus clear that by increasing the expression of cyclase
5 early in the fermentation of penicillin V and maintaining an elevated level of the activity the production of penicillin is started earlier and the accumulated yields are increased.

As will be apparent to those skilled in the art in the light of
10 the foregoing disclosure, many alterations and modifications are possible in practice of this invention without departing from the spirit or scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the following claims.

PATENT CLAIMS

1. A process for the production of a secondary metabolite comprising
 - 5 i) fermentation of a microorganism capable of producing said secondary metabolite, and
 - ii) recovering said metabolite in substantially pure form, wherein said microorganism has been modified in a manner whereby the expression of one or more of the DNA sequences
- 10 coding for (a) peptide(s), (a) protein(s) or (an) enzyme(s), involved in or interfering with the biosynthetic pathway of said secondary metabolite, is regulated differently from the regulation of said DNA sequence(s) in the original microorganism.
- 15
2. The process according to claim 1, wherein said expression is initiated at an earlier fermentation stage in comparison to the expression in the original microorganism.
- 20 3. The process according to claim 1, wherein the expression level of said peptide(s), protein(s) or enzyme(s) is increased at an earlier fermentation stage in comparison to fermentation of the original microorganism.
- 25 4. The process according to claim 1, wherein said expression is initiated at a later fermentation stage in comparison to the expression in the original microorganism.
5. The process according to claim 1, wherein the expression
- 30 level of said peptide(s), protein(s) or enzyme(s) is increased at a later fermentation stage in comparison to fermentation of the original microorganism.
6. The process according to any of the claims 1 to 5, wherein
- 35 said expression is maintained throughout the fermentation.
7. The process according to any of the claims 1 to 6, wherein said expression is synchronized to the expression of other

genes encoding enzymes belonging to the same biosynthetic pathway.

8. The process according to any of the claims 1 to 7, wherein
5 the microorganism used for the fermentation is immobilized.

9. The process according to any of the claims 1 to 8, wherein
the fermentation is continuous.

10 10. The process according to any of the claims 1 to 9, wherein
said modification is accomplished by substitution of the
promoter region(s) regulating the expression of said DNA
sequence(s).

15 11. The process according to any of the claims 1 to 10, which
is a process for the production of an antibiotic, clavulanic
acid or indole dihydrodiol.

12. The process according to claim 11, which is a process for
20 the production of an antibiotic selected from the group
comprising penicillins, cephalosporins, cephamycins, mono-
bactams, chloramphenicol, erythromycin, streptomycin, and
nocardicins.

25 13. The process according to claim 12, which is a process for
the production of penicillin.

14. The process according to claim 13, which is a process for
the production of penicillin G or penicillin V.

30

15. The process according to any of the claims 12 to 14,
wherein said DNA sequence is the DNA sequence coding for the
isopenicillin N synthase (cyclase or IPNS) enzyme.

35 16. The process according to claim 15, wherein the IPNS
promoter is substituted by the promoter (ATp) regulating the
expression of the acyl-CoA:6-amino penicillanic acid
transferase enzyme (AT).

17. A process for the production of a microorganism capable of producing a secondary metabolite, wherein an original microorganism is modified in a manner whereby the expression of one or more of the DNA sequences coding for (a) peptide(s), (a)
5 protein(s) or (an) enzyme(s), involved in or interfering with the biosynthetic pathway of said secondary metabolite, is regulated differently from the regulation of said DNA sequence(s) in the original microorganism.

10 18. The process according to claim 17, wherein said original microorganism is modified in a manner whereby said expression is initiated at an earlier fermentation stage in comparison to the expression in the original microorganism.

15 19. The process according to claim 17, wherein said original microorganism is modified in a manner whereby the expression level of said peptide(s), protein(s) or enzyme(s) is increased at an earlier fermentation stage in comparison to fermentation of the original microorganism.

20

20. The process according to claim 17, wherein said original microorganism is modified in a manner whereby said expression is initiated at a later fermentation stage in comparison to the expression in the original microorganism.

25

21. The process according to claim 17, wherein said original microorganism is modified in a manner whereby the expression level of said peptide(s), protein(s) or enzyme(s) is increased at a later fermentation stage in comparison to fermentation of
30 the original microorganism.

22. The process according to any of the claims 17 to 21, wherein said original microorganism is modified in a manner whereby the expression is maintained throughout the
35 fermentation.

23. The process according to any of the claims 17 to 22, wherein said original microorganism is modified in a manner

whereby the expression of the genes is synchronized to the expression of other genes belonging to the same biosynthetic pathway.

5 24. The process according to any of the claims 17 to 23, wherein the microorganism used for the fermentation is immobilized.

25. The process according to any of the claims 17 to 23, where-
10 in the fermentation is continuous.

26. The process according to any of the claims 17 to 25, where-
in said original microorganism is modified by substitution of
the promoter(s) regulating the expression of said DNA sequen-
15 ce(s).

27. The process according to any of the claims 17 to 26, where-
in said original microorganism belongs to a genus from the
group comprising *Penicillium*, *Cephalosporium*, *Aspergillus*,
20 *Nocardia*, *Streptomyces*, *Escherichia*, *Bacillus*, *Pseudomonas*,
Cercospora, *Micromonospora*, other *Eubacteria*, other
Actinomycetes or filamentous fungi.

28. The process according to any of the claims 17 to 27,
25 wherein said original microorganism is a microorganism capable
of producing an antibiotic, clavulanic acid or indole
dihydrodiol.

29. The process according to claim 28, wherein said original
30 microorganism is a microorganism capable of producing an
antibiotic selected from the group comprising penicillins,
cephalosporins, cephamycins, mono-bactams, chloramphenicol,
erythromycin, streptomycin, and nocardicins.

35 30. The process according to claim 28 or 29, wherein said
original microorganism belongs to a species from the group
comprising *Penicillium chrysogenum*, *Acremonium chrysogenum*,

Aspergillus nidulans, *Nocardia lactamdurans*, *Streptomyces clavuligerus* and *Pseudomonas putida*.

31. The process according to claim 30, wherein said original
5 microorganism is a microorganism capable of producing penicillin.

32. The process according to claim 31, wherein said original
microorganism is a microorganism capable of producing
10 penicillin G or penicillin V.

33. The process according to claim 32, wherein said DNA se-
quence is the DNA sequence coding for the isopenicillin N
synthase (cyclase or IPNS) enzyme.

15

34. The process according to claim 33, wherein the IPNS
promoter is substituted by the promoter regulating the
expression of the acyl-coenzyme A:6-amino penicillanic acid
transferase enzyme.

20

35. A DNA construct comprising gene(s), involved in the
secondary metabolism, which is expressed under the control of
a promoter different from the original promoter.

25 36. The DNA construct according to claim 35, wherein the
promoter is a promoter of any of the genes in the secondary
metabolism of a microorganism.

37. The DNA construct according to claim 35 or 36, wherein said
30 gene(s) encode(s) peptide(s), protein(s) or enzyme(s) involved
in the biosynthesis of a β -lactam, clavulanic acid or indole
dihydrodiol.

38. The DNA construct according to any of the claims 35 to 37,
35 wherein the gene(s) encoding peptide(s), protein(s) or
enzyme(s) of the penicillin biosynthesis.

39. The DNA construct according to any of the claims 35 to 38, wherein the gene is the gene encoding the isopenicillin N synthase (cyclase or IPNS).

5 40. The DNA construct according to claim 35 to 39, wherein the promoter is a promoter of genes encoding peptide(s), protein(s) or enzyme(s) involved in the β -lactam biosynthesis.

41. The DNA construct according to claim 40, wherein the
10 promoter is a promoter of said gene(s) involved in the penicillin biosynthesis.

42. The DNA construct according to claim 41, wherein the promoter is the promoter of the acyl-CoA:6-amino penicillanic
15 acid transferase (AT).

43. The DNA construct according to claim 35 to 42 comprising the IPNS structural gene and the terminator, which is expressed under control of an AT promoter.

20

44. The DNA construct according to claim 35, wherein the promoter is a promoter of any of the genes involved in the primary metabolism of a microorganism.

25 45. The DNA construct according to claim 35, wherein the promoter comprises an operable part of the promoter DNA sequence(s) according to claim 36 to 44.

46. A vector or transformation vehicle comprising a DNA
30 construct according to any of the claims 35 to 45.

47. A vector or transformation vehicle comprising a DNA construct according to any of the claims 35 to 45, wherein said DNA construct is operably linked to a sequence encoding a
35 targeting signal.

48. A vector or transformation vehicle comprising a DNA construct according to any of the claims 35 to 45, wherein said vector comprise an operably linked phleomycin resistant unit.

5 49. A vector or transformation vehicle comprising a DNA construct according to any of the claims 35 to 45, wherein said vector is pUC19.

50. A microorganism capable of producing a secondary
10 metabolite, which microorganism has been modified in a manner whereby the expression of one or more of the DNA sequences coding for (a) peptide(s), (a) protein(s) or (an) enzyme(s), involved in or interfering with the biosynthetic pathway of said secondary metabolite, is regulated differently from the
15 regulation of said DNA sequence(s) in the original microorganism.

51. The microorganism according to claim 50, produced by any of the processes of any of the claims 17 to 34.

20

52. The microorganism according to claim 50 or 51, wherein a vector or DNA construct of any of the claims 35 to 45 has been used for said modification.

25 53. The microorganism according to any of the claims 50 to 52, wherein said microorganism belongs to a genus from the group comprising *Penicillium*, *Cephalosporium*, *Aspergillus*, *Nocardia*, *Streptomyces*, *Escherichia*, *Bacillus*, *Pseudomonas*, *Cercospora*, *Micromonospora*, other *Eubacteria*, other *Actinomycetes* or
30 filamentous fungi.

54. The microorganism according to any of the claims 50 to 53, wherein said microorganism is a microorganism capable of producing an antibiotic, clavulanic acid or indole dihydrodiol.

35

55. The microorganism according to claim 54, wherein said microorganism is a microorganism capable of producing an antibiotic selected from the group comprising microorganisms

capable of producing penicillins, cephalosporins, cephamycins, mono-bactams, chloramphenicol, erythromycin, streptomycin, and nocardicins.

5 56. The microorganism according to claims 54 and 55, wherein said microorganism belongs to a species from the group comprising *Penicillium chrysogenum*, *Acremonium chrysogenum*, *Aspergillus nidulans*, *Nocardia lactamdurans*, *Streptomyces clavuligerus*, *Pseudomonas putida* and *E. coli*.

10

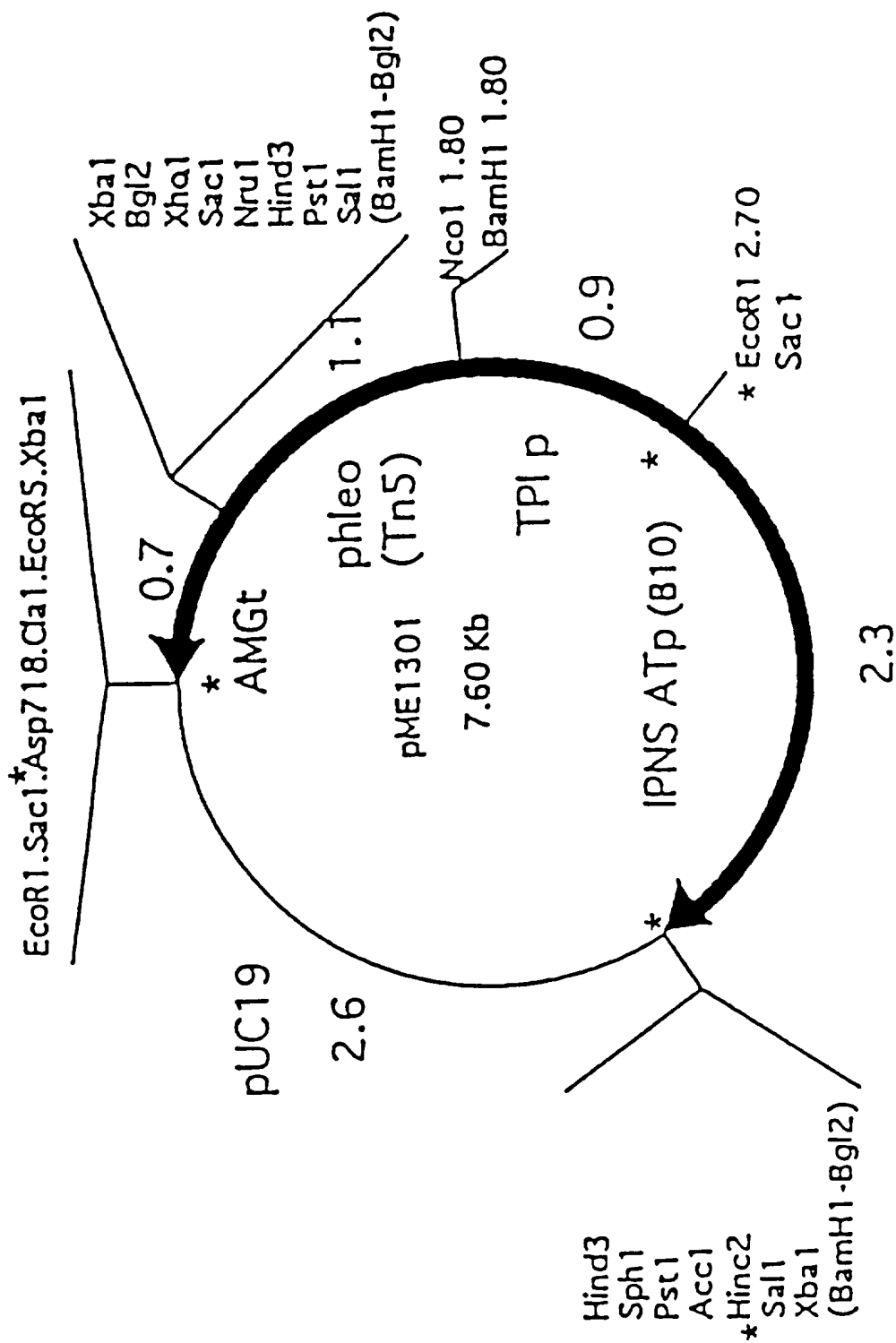
57. A secondary metabolite produced by a process according to any of the claims 1 to 16.

15 58. The secondary metabolite according to claim 57, wherein said metabolite is an antibiotic, clavulanic acid or indole dihydrodiol.

59. The secondary metabolite according to claim 57 or 58, wherein said metabolite is an antibiotic selected from the 20 group comprising microorganisms capable of producing penicillins, cephalosporins, cephamycins, mono-bactams, chloramphenicol, erythromycin, streptomycin, and nocardicins.

60. The secondary metabolite according to any of the claims 57 25 to 59, wherein said metabolite is penicillin.

61. The secondary metabolite according to claim 53 to 56, wherein said metabolite is penicillin G or penicillin V.



* Ligation site
Figure 1

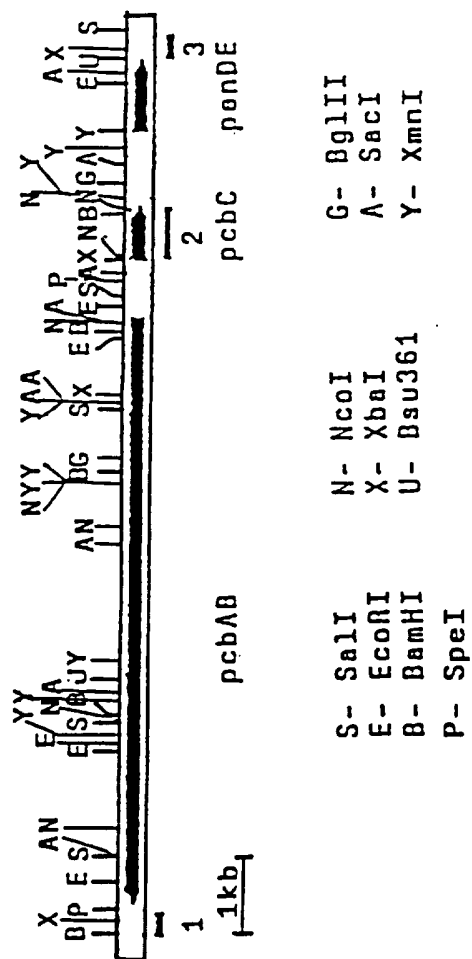


Figure 2

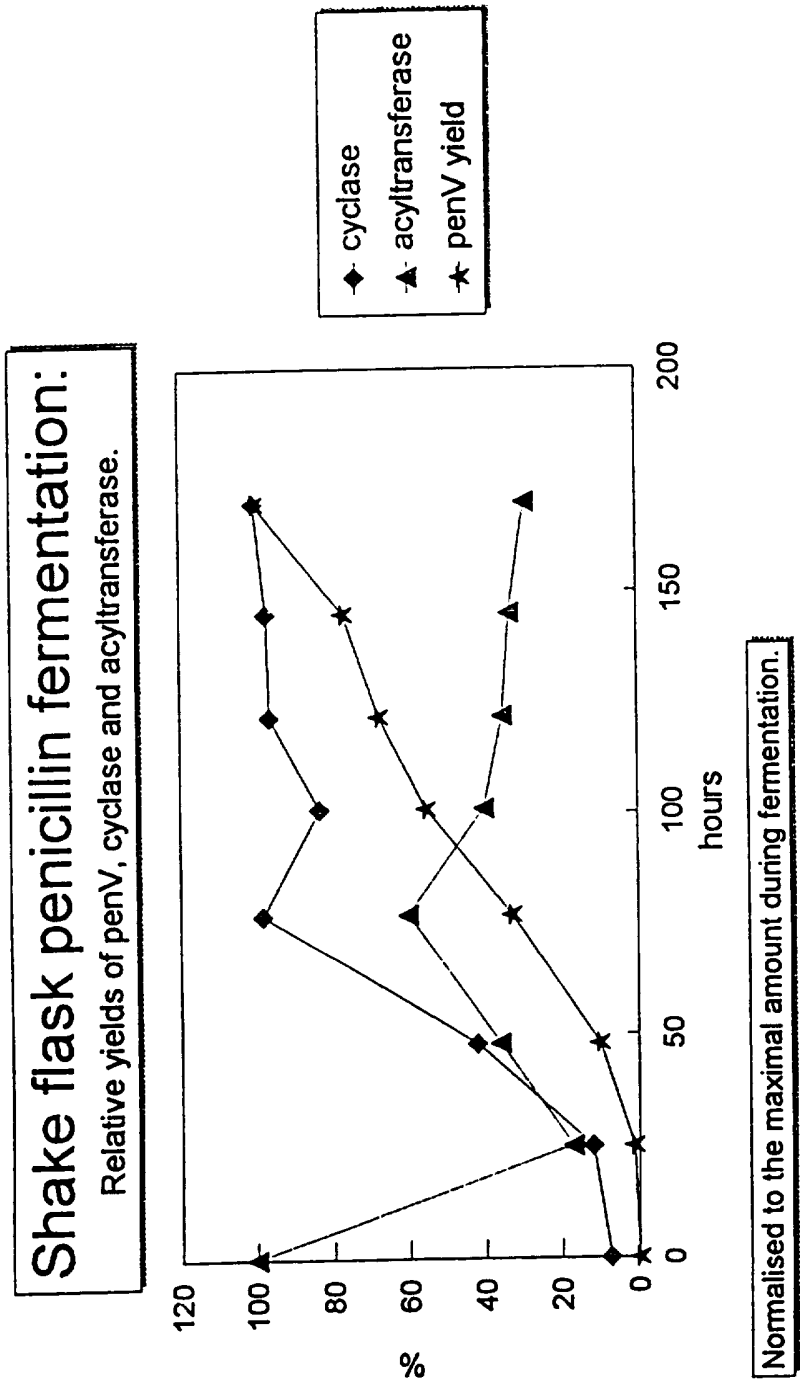


FIGURE 3

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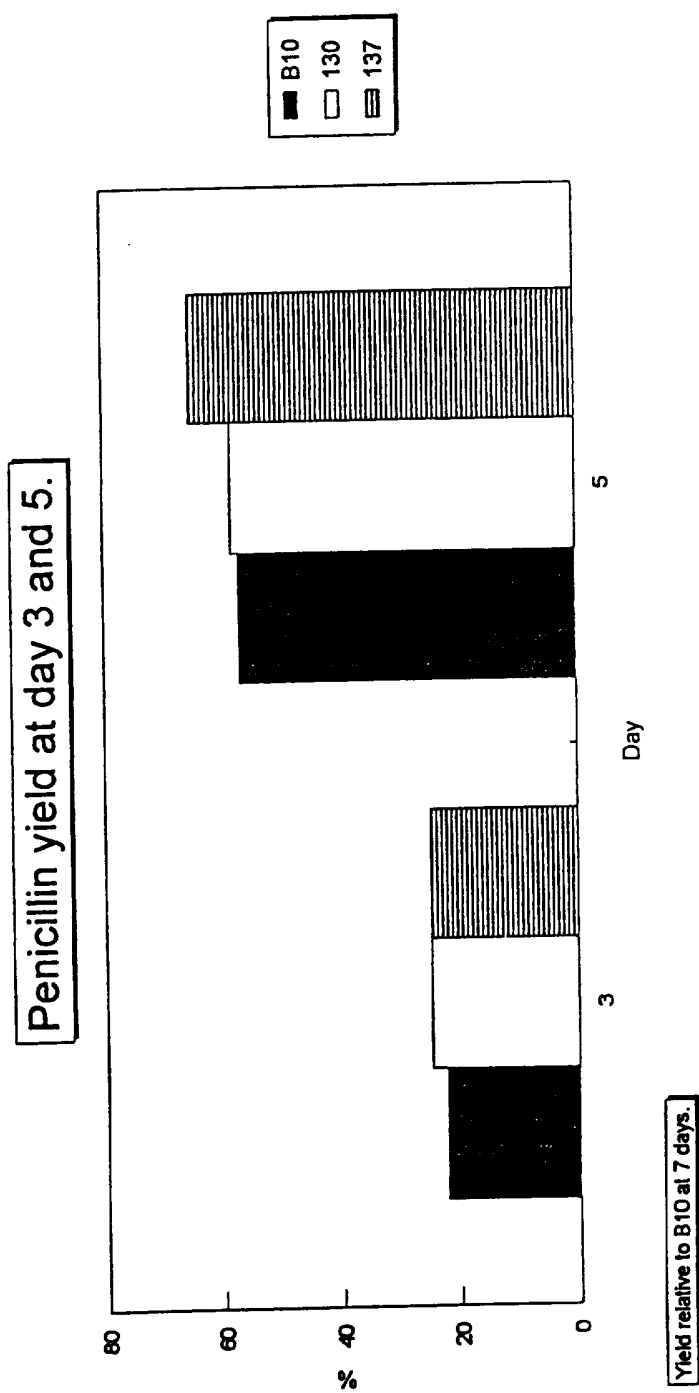


FIGURE 4

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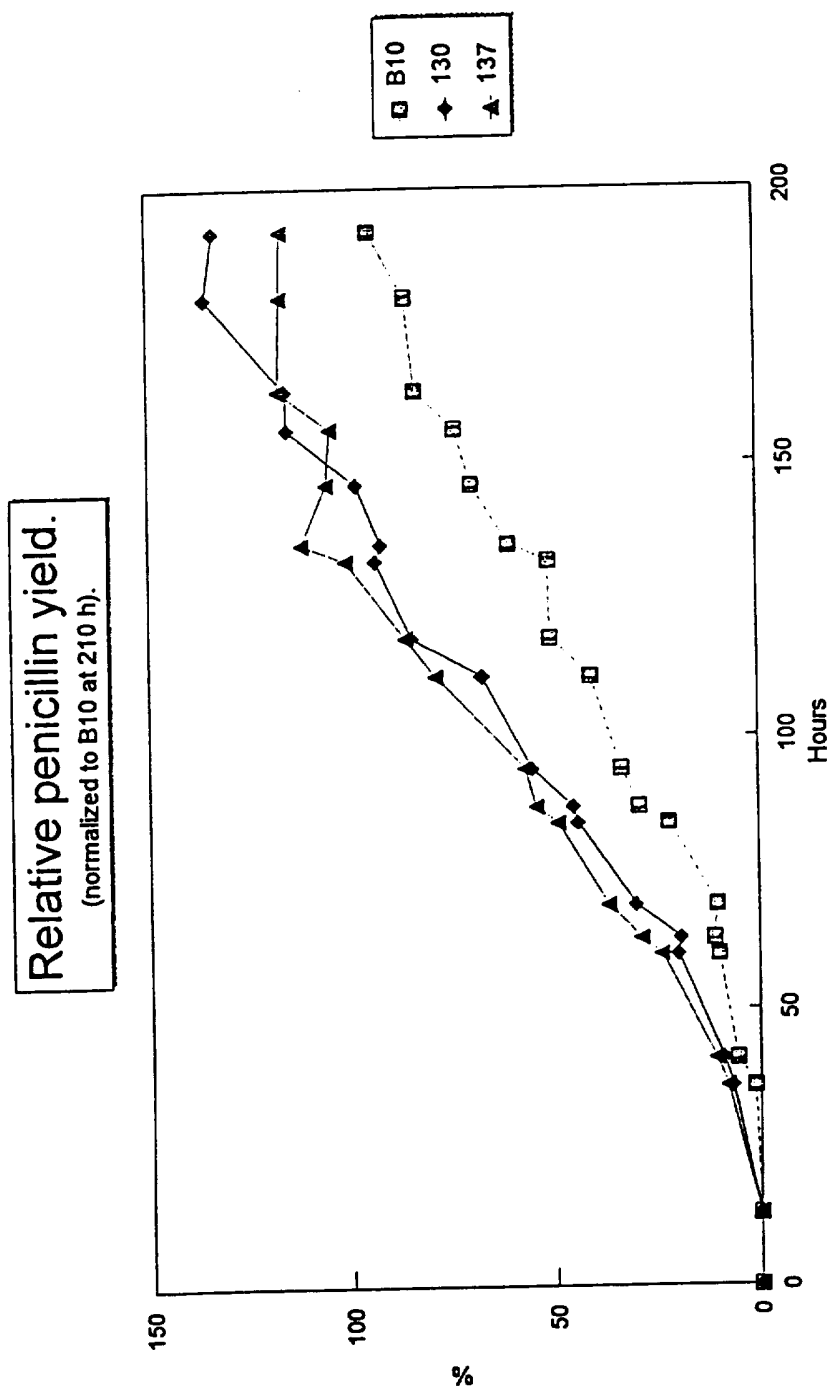


FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00386

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/52, C12P 1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12P, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CA, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"The Merck Index, Eleventh edition", 1989, see page 1123 "Penicillin G Potassium" --	57-61
X	"The Merck Index, eleventh edition", 1989, page 1125 "Pennyroyal" --	57-61
A	EP 0260762 A2 (GIST-BROCADES N.V.), 23 March 1988 (23.03.88) --	1-56
A	EP 0379428 A1 (TRANSGENE S.A.), 25 July 1990 (25.07.90) --	1-56



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

28 January 1996

Date of mailing of the international search report

05 02- 1996

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00386

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Ciba Found Symp, Volume 171, 1992, Arnold L. Demain, "Microbial secondary metabolism: a new theoretical frontier for academia, a new opportunity for industry", page 3 - page 16, discussion 16-23 -- -----	1-56

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00386

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-5, 17-21, 50
because they relate to subject matter not required to be searched by this Authority, namely:

The formulations "regulated differently from", "at an earlier termentation stage" and "at a later termentation stage" do not define the matter sought. The claims are not clear and concise. See Art 6.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

05/01/96

International application No.
PCT/DK 95/00386

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0260762	23/03/88	SE-T3- 0260762 AU-B,B- 614267 AU-A- 7845187 DE-D,T- 3788764 ES-T- 2061483 IE-B- 61984 JP-A- 63192378 NO-C- 173451	29/08/91 24/03/88 23/06/94 16/12/94 14/12/94 09/08/88 15/12/93
EP-A1- 0379428	25/07/90	DE-D,T- 69003693 FR-A,B- 2642768 JP-A- 3180174	24/02/94 10/08/90 06/08/91