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HOW TO INCREASE THE YIELD OF INDUCIBLE ENZYMES IN MICROORGANISMS

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Abstract: The methods of increasing the yield of inducible enzymes (primarily) are listed in the paper. A number of methods are based on optimizing the cultivation conditions of the selecting producer, primarily by change of the growth medium composition; among the rest by application of inducers and by exclusion of components which cause catabolic repression or end-product repression. The other group of methods includes those based on obtaining and selecting mutants, both those constitutive and resistant to catabolic repression and those in which the increase of yield is accomplished by other mechanisms.

1 Introduction

Enzymes are biologic catalyzers which are becoming more and more important because of their application in medicine, analytics, synthesis of organic compounds, systems of energy conversion and many branches of industry. One of the main reasons why enzymes do not compete even more with other, alternative, processes, is their at average relatively high price. Therefore, one of the main tasks of industrial enzymology is to make the enzyme obtaining process as economical as possible. From the enzymes which have found their application in different fields only about 4 % are from various plant species and only about 8 % are from different animal species. All other enzymes are from microorganisms, more than a half from fungi and yeasts and more than one third from bacteria. Reasons for this are primarily in the fact that, as different from plants and animals, in cells of microorganisms the quantities of produced enzyme may be easily increased by selection, by choice of growth medium as well as by genetic manipulations. In catabolic enzymes the yield can be increased thousandfold and in biosynthetic enzymes several hundredfold. Another advantage of microorganisms is that they are economical which is due to short fermentation cycles and cheap nutrient media [1]. Enzymes in microorganisms may be extracellular or intracellular depending on whether they excrete or not into outer medium, and inducible, partly inducible or constitutive depending on whether the presence of a specific substance, inducer, is necessary for their biosynthesis. Yield of all enzymes can be increased by choice of strain, by optimizing the cultivation process parameters (pH, temperature, aeration and nutrient medium composition) and selection of highest point of the growth cycle [2]. It will be discussed here how to make the process of obtaining inducible enzymes more economical

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by application of the results of research of microorganisms genetics to increase enzymes yield.

2. APPLICATION OF INDUCERS

Regulation of biosynthesis of enzymes and other proteins is carried out in microorganisms for the most part at the level of transcription, by induction and repression as main mechanisms which may have positive and negative control [1-9]. The product of given operon regulatory gene (protein repressor), by binding itself to the operator blocks the moving of RNA polymerase from promoter along DNA matrix toward terminator thus preventing the transcription process of structural genes. Gene expression occurs only when the inducer is present in the system; the inducer binds itself to repressor and builds a repressor-inducer complex which either no longer has or has a reduced affinity toward the operator. According to this mechanism the biosynthesis is carried out of enzyme β-galactosidase in many producers, glucose isomerase in *Streptomyces phaeochromogenes* [3], amidase in *Pseudomonas aeruginosa* [10], enzyme galoperon in *E. coli* and also the dextransucrase in *Leuconostoc mesenteroides* [11,12] and dextranase in many fungi, yeasts and bacteria [13,14]. The role of inducer is most often played by substrates of the given enzyme, but also by substrate analogs (Table 1) and reaction products (Table 2).

Table 1. Substrates and Substrate Analogs as Enzyme Inducers

Enzyme	Substrate	Substrate-analog-inducer
Penicillin β-lactamase	Penicillin	Methicillin
Aliphatic amidase	Acetamide	N-methylacetamide
Cellulase	Cellulose	Sophorose
Maleate-fumarate cys-trans isomerase	Maleic acid	Malonic acid
Tyrosinase	L-tyrosine	D-tyrosine, D-phenylalanine
β-Galactosidase	Lactose	Isopropyl-β-D-thiogalactoside

Table 2. Reaction Products as Enzyme Inducers

Enzyme	Microorganism	Substrate	Product-inducer
Dextranase	*Penicillium*	Dextran	Isomaltose
Pullulanase	*Klebsiella aerogenes*	Pullulan	Maltose
Amylase	*Bacillus stearothermophilus*	Starch	Maltodextrins
Amyloglucosidase	*Aspergillus niger*	Starch	Maltose, isomaltose
Lipase	*Geotrichum candidum*	Lipids	Fatty acids
Histidine ammonia-lyase	*Klebsiella aerogenes*	Histidine	Urocanic acid
Urea carboxylase	*Saccharomyces cerevisiae*	Urea	Alophanic acid
Pectinolytic enzymes	*Acrocilindrium*	Pectin	Galacturonic acid
Tryptophan oxygenase	*Pseudomonas*	Tryptophan	Kynurenine

Enzyme substrates are usually very good inducers, while the reaction products are most often much weaker. End-product repression and/or catabolic repression which reduce the

enzyme yield are usually present in reaction products. We usually encounter enzyme substrates as natural inducers. The exceptions are the substrates of enzyme β-galactosidase and histidine ammonia-lyase which are not inducers of these enzymes. Lactose is, in fact, a weak antiinducer but by action of small quantities of consitutive β-galactosidase it is converted into glucose and galactose which in parallel reaction transform into allolactose (6-O-β-D-galactopyranosil-D- glucose) and galactosido-glycerol which are very strong inducers [5,15].

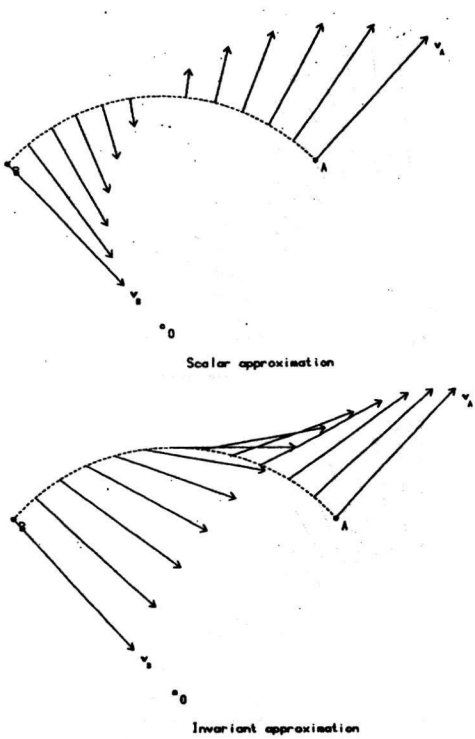

Fig. 1

For the purpose of increasing enzyme production it is useful to test inducing effects of different substrate analogs some of which may have even 1000-times stronger inducing action than the substrate itself, where enzyme may reach up to 10 % of the total dry mass of cells [1,16]. Inducers of enzyme β-galactosidase are many compounds with galactopyranose ring where aglicone structure may be very different. Inducing activity increases on substitution of O-galactose residue with S-galactose residue and especially so if aliphatic groups are linked to S. The two most efficient inducers of β-galactosidase are isopropyl-β-D-thiogalactoside and methyl-β-D-thiogalactoside which do not have affinity toward the enzyme and do not hydrolyze by its action. Melibiose (6-O-α-D-galactopyranosyl- D-glucose)

is not a substrate of β-galactosidase and compared to lactose it is twice potent as an inducer. Phenylethyl-β-D-thiogalactoside has maximum affinity toward this enzyme, but has a weak inducing action and does not hydrolize. o- and p-nitrophenyl-β-D-galactoside and o-nitrophenyl- α-L-arabinoside are not inducers of β-galactosidase, but have affinity toward the enzyme and toward lac-repressor and act as antiinducers and substrates at the same time [17,18]. Non-metabolizing inducers are usually more efficient because: a) their concentration does not decrease during cultivation, and b) reaction products are not formed which may act as repressors.

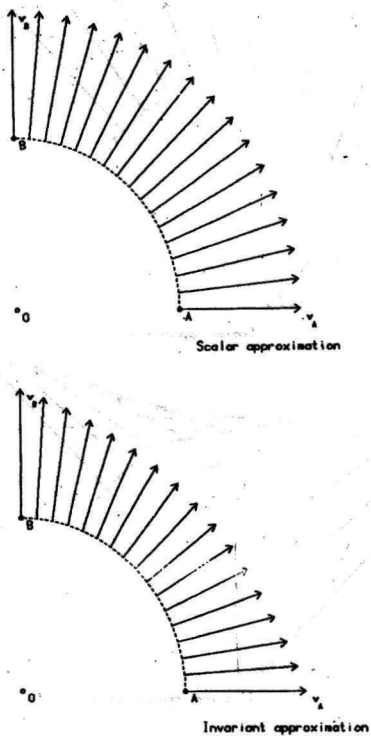

Fig. 2

Beside the already existing substrate analogs it is often necessary to synthetize new ones. As a rule, synthesis should lead to formation of substrate analogs, i.e. analogs of natural inducers which will not so readily utilized by microorganisms. Dipalmitate isomaltose is 733 times better inducer of dextranase than isomaltose [19] and ketodextran is 75 times better than dextran [20]. In inducers with several functional groups it could be expected that with increase of modification degree up to a certain degree, the inducing action will also increase but with further modification it will start to decrease. Beside the degree of modification the structure of substituent is also important. Previous observations suggest that hydrophobicity of substituents increases inducing action while electronaccep-

tor groups act reversely. In aliphatic amidase the substrate acetamide is an inducer at the same time, N-methyl-acetamide is a much better inducer while the cyanoacetamide is an antiinducer. Since an increase in degree of modification leads to reduction of affinity of modified substrate toward enzyme, this reduces the possibility of its utilization by microorganisms and in pronounced cases addition of limited quantities of new carbon source leads to even greater increase of enzyme production [20].

Another approach which often leads to formation of hyperproducers is obtaining "constitutive" mutants which without an inducer form inducible enzymes. Selection of constitutive mutants from mutagenetic population of microorganisms can be carried out as follows:

A. On growth in hemostat with limiting of substrate-inducer. Example: on cultivation in hemostat with low concentration of lactose it comes to selection of mutants of *E. coli* wich form, without an inducer, β-galactosidase in quantity of 25% of its total proteins [10].

B. Alternative repeating of growth on medium with and without an inducer leads to enrichment of microorganisms population with constitutive mutants.

C. By using substances which are good substrates and bad inducers. Examples:

a) By using o-nitrophenyl-α-D-arabinoside for β-galactosidase in *E. coli* or

b) By using acrylamide as the only source of nitrogen for obtaining constitutive mutant producers of aliphatic amidase in *P. aeruginosa.*

D. By using antiinducers along with enzyme substrate, e.g., o-nitrophenyl- β-D-fucoside with melibiose or lactose.

E. By visual detection without enrichment if the frequency of mutations is high enough, e.g.:

a) Spraying with o-nitrophenyl-β-D-galactoside colonies of *E. coli* grown on solid medium with glycerol as carbon source. Only colonies which form β-galactosidase without an inducer will be coloured yellow.

b) With 5-bromo-4-chloro-3-indolyl-β-D-galactoside, which is a substrate but not an inducer for β-galactosidase, in the presence of constitutive β-galactosidase on media without lactose, produces blue colour from 5-bromo-4-chloro-indigo, which does not diffuse into the medium nor into colonies of producer [9].

3. PREVENTION OF REPRESSION BY END-PRODUCT

In anabolic enzymes regulation is achieved by repression mechanism: regulatory gene product is proteine aporepressor which is capable of being bound to operator and of blocking gene expression only if corepressor is bound to it; and corepressor is just an enzyme reaction product.

Regulation of catabolic enzymes biosynthesis is usually double: through induction and repression. Repression is being carried out by metabolites which are utilized quickly and constitutively (catabolic repression) or by final products of given enzymes reaction, or with both simultaneously. For example, in *Proteus rettgeri* the urease production is induced by urea and repressed by ammonia and is at the same time subject to catabolic repression.

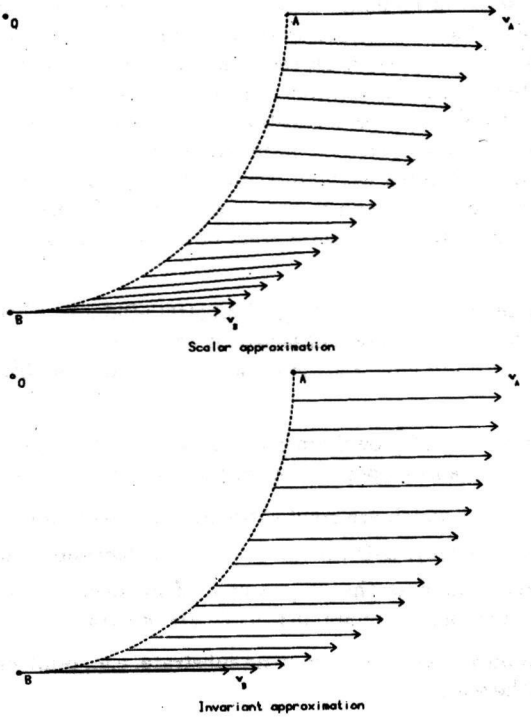

Fig. 3

In fungi of genus *Neurospora*, nitrate reductase is induced by nitrate and repressed by ammonia.

One of the basic ways of preventing repression by end-product is carrying out the limitation in reaction products when choosing growth medium. Thus the production of proteolytic enzymes is considerably increased when growth medium does not contain aminoacids. If in *Bacillus licheniformis* glucose or malate are used as a carbon source instead of glutamate or caseine hydrolyzate, then 20 times greater production of glutamate dehydrogenase is obtained [21]. Limitation of phosphate in *Aspergillus quercinus* increases the yield of nuclease and phosphatase by 30 to 50 times [22], and in *E. coli* the yield of alkaline phosphatase is increased from zero to about 5% of the total cell proteins.

The second method is application of inhibitor of the given enzyme, which prevents the formation of the end-product by acting on the substrate which is a constituent of a nutrient medium. Thus the production is considerably increased and the inhibitor is removed during the enzyme purification.

The third method is limitation of access of growth factor to auxotrophic mutant. Thus, for example, partly starving on thiamine leads to 1500 times greater production of thiamine phosphate pyrophosphorylase, one of the enzymes of thiamin biosynthesis. Instead of limitations in the quantity of nutrient constituent there could also be used slowly utilizing

derivates of necessary final products; thus, for example, cultivation of uracil auxotroph on dihydroorotic acid leads to a 1000-fold increase yield of aspartatecarbamoyltransferase, or 7% of the total proteins in cells [23].

Enzyme yield can be greatly increased also by application of mutants resistant to end-product repression. These are so called "regulatory" mutants which behave as constitutive ones, like those ones that do not require an inducer. Most probably, either the regulatory genes are so changed in them that they produce defective aporepressor, or the mutated operator does not bind the formed aporepressor-corepressor complex. Selection of these mutants can be realized:

A. According to resistance to toxic analogs of end-products. For example, forming of enzyme set may be liberated from repression control by ammonia as an end-product, if methylamin resistant mutants are obtained.

B. By reversion of auxotrophic mutants. Example: revertant obtained from mutant with defective hemoserine dehydrogenase gives 3 times higher yield of this enzyme in comparison with starting prototrophic microorganism [10].

C. By sporulation mutants. In bacilli, forming of protease is the same as sporulation inhibited by aminoacids [24,25]. Mutants, capable of sporulating in the presence of aminoacids, can easily be isolated by heating method. Thus obtained *Bacillus cereus* mutants produce about 10 times more protease.

D. By direct visualization without enrichment. Example: colonies of mutants constitutive to enzyme phosphatase may be detected after growth in presence of high concentrations of phosphates and spraying with p-nitrophenylphosphate. Constitutive mutants will be coloured yellow, while the repressible colonies remain white.

4. PREVENTION OF CATABOLIC REPRESSION

Growth of most microorganisms on quickly utilizing carbon sources induces repression of synthesis of inducible and constitutive, first of all catabolic, enzymes. Significance of catabolic repression is all the greater since most of industrially important and promising enzymes are apt to this type of regulation (Table 3). Carbon source which in

most enzymes induces catabolic repression is glucose but such repressor role play many other compounds, often with even stronger action. There is evidence that cAMP controlled operon promoters form hierarchy in different affinity to cAMP-CAP complex and that concentration of this complex increases proportionally to increase of cAMP concentration as a response to carbon fasting. It is also known that small concentrations of glucose (50 μM) not only do not act repressively but also anhance synthesis of enzymes.

Increase of enzyme yield by prevention of catabolic repression is carried out in the following ways:

A. By exclusion from growth medium of those carbon sources which cause catabolic repression. Examples:

a) By cultivation of *B. stearothermophilus* on glycerol instead of fructose, the yield of extracelullar α-amylase increases by more than 25 times.

Table 3. Catabolic Repression of Enzymes

Enzyme	Organism	Repression inducing carbon source
α-Amylase	Bacillus licheniformis	Glucose, mannite
	B. amyloliquefaciens	Glucose
	B. stearothermophilus	Fructose
Cellulase	Trichoderma viride	Glucose, glycerol, starch, cellobiose
	Pseudomonas fluorescens	Galactose, glucose, cellobiose
Protease	Bacillus licheniformis	Glucose, mannite
	B. megaterium	Glucose
	Candida lipolitica	Glucose
Invertase	Neurospora crasa	Mannose, glucose, fructose, xylose
Amyloglucosidase	Endomycopsis bispora	Starch, maltose, glucose, glycerol
Aliphatic amidase	P. aeruginosa	Succinate
Methylene hydroxylase	Artrobacter sp.	Acetate

b) If *Pseudomonas fluorescens var.cellulosa* is cultivated on mannose instead of galactose the production of cellulase is increased by 1500 times.

If for economic reasons cultivation is carried out on carbon sources which cause catabolic repression, it can be considerably reduced by limitation of growth spead in hemostat. Slow bringing of glucose to *P. fluorescens var.cellulosa* increases production of cellulose almost 200 times.

B. In cases where fast decomposition of inducer leads to formation of products which cause catabolic repression, like in enzyme invertase, cellulase, dextranase and β-galactosidase, production of enzymes can be increased:

a) By slow introduction of inducer-substrate into the system.

b) By application of slowly metabolizing analog of inducer as a carbon source.

c) By using slowly utilizing derivate of inducer, such as esters. If instead of sucrose its monopalmitate is used as an inducer, 80 times greater production of invertase is obtained [19].

C. Utilization of mutants resistant to catabolic repression is useful for several reasons. Greater yields of enzymes are almost always obtained with them and in some cases the yields are extremely high; e.g. in mutants of yeast, resistant to catabolic repression, which produce invertase in the quantity of almost 2% of its cell proteins. They often form and excrete extracellular enzymes in early stages of growth cycle in periodic cultures in comparison with initial strains, thus increasing the economy of production. These mutants allow utilization of cheap fermentation media which often contain bigger quantities of glucose.

Many of these mutants have modified ways of glucose metabolism and therefore much slower glucose utilization which leads to liberation of multitude of enzymes sensitive to catabolic repression. However, in a number of mutants resistance is bound specifically to one enzyme or one metabolic way. Selection of these mutants can be realised in the following way:

a) By alternative repeating of growth on media with and without glucose. *E. coli* mutants do not show lag period when transferred from glucose to medium containing lactose, maltose, succinate or acetate, and in that way they are enriched.

b) By utilizing substrates of catabolically repressed enzyme as the sole nitrogen source, e.g.:

1. By transfer of *Aerobacter aerogenes* into medium with glucose and histidine as the sole nitrogen source, the mutants are selected which produce histidine ammonia-lyase in the presence of glucose.

2. Mutants of bacterium *Salmonella tiphimurium* which is resistant to catabolic repression of enzyme proline oxidase are obtained by cultivation on glucose-proline agar.

3. Only the mutants of *P. aeruginosa* which are resistant to catabolic repression are capable of growth on medium with lactamide (a good inducer but bad substrate of amidase) in the presence of succinate. Such mutants form intracellular amidase in the quantity of up to 10% of total cell proteins [26].

5. GENETIC CONSTRUCTION OF MICROORGANISMS IN VIVO AND IN VITRO

Genetic construction in vivo consists of formation and isolation of mutants and utilization of different methods of exchange of genetic information of alive microbial cells. Beside the already mentioned methods, increase of yield of inducible enzymes can be achieved by application of such universal methods.

Formation of mutants by utilization of physical (raised temperature, quick neutrones, UV-, X- and γ-rays), chemical (base analogs, alkylating and intercalating agents, HNO_2, $HONH_2$ and others) and biologic mutagene factors can permit not only the increase of yield but also improvement of enzyme properties. Isolation of mutants (morphological, biochemical and physiological) with the best properties is carried out by different methodes of selection [3,8,27,28]. Explanation of increase of enzyme yield in mutants and revertants is, beside the explanation presented in sections 2, 3 and 4, also in the following:

a) mutations on promoter which lead to greater affinity to RNA-polymerase and to greater frequency of expression;

b) mutations which lead to increased secretion;

c) mutations which lead to reduced production of proteolytic enzymes and

d) mutations which lead to reduced production of inhibitors, repressors etc.

Transfer of genes from one microorganism into another can be realised by classical methods of conjugation, transformation and transfection.

Hybrids with different properties can be obtained in relatively simple way by fusion of protoplasts in grampositive bacteria, yeast and micellar fungi [3,8,24,29,30]. Fusion of protoplasts, obtained by removing cell membrane under the action of enzyme in isotonic solution, is carried out in the presence of polyethylene glycol and Ca^{2+}. If the chromosomes are basically homologs then it often comes to their recombination in the fusant, and if they are incubated in the adequate medium then the cell membrane is formed again and they

revert to normal form of a hybride microorganism. Thus e.g., by fusion of protoplast of *Bacillus subtilis* and *Cellulomonas uda* fusants were obtained capable of adopting cellulose as the only carbon source, which means that genes responsible for synthesis of cellulase were transferred into cells of *B. subtilis*.

Beside the classic mutagenesis the methods of site-directed mutagenesis for obtaining enzymes of exactly defined (changed according to wish) structure and with improved properties [8,31-33] are also used nowdays.

Genetic construction of microorganisms in vitro, known under the names of technologies of recombinant DNA, molecular cloning, gene cloning or genetic engineering, consists in linking the fragments of DNA in vitro and in subsequent introduction of such new "recombinant" genetic structures in the alive cell [5,8,28,32-36]. Beside many other advantages, this method enables formation of given enzyme in producers which otherwise do not produce it, if it is more favourable; also, obtaining of bigger yields on the principle that yield of enzyme depends not only on frequency of expression under the given cultivation conditions, but also on the number of copies of given gene.

6. CONCLUSION

In microorganisms, as the most promising producers of enzyme, the yield of enzyme can be manyfold increased (even by more than thousand times), by application of different methods: a) by choosing the most productive strain; b) by optimization of cultivation process parameters and choosing the highest point of growth cycle; c) by utilizing inducers; d) by exclusion from the growth medium of those substances which cause catabolic repression or repression by end-product; e) by obtaining and selecting constitutive mutants, mutants resistant to catabolic repression and mutants which through other mechanisms give greater production of enzymes; f) by using methods of fusing protoplasts; g) methods of cloning genes; etc. The choice of individual methods is conditioned by the kind of regulation of gene expression of given enzyme. Increase of yield is one of the main factors which should make the enzyme obtaining process more economic and provide better competitiveness for enzymes.

Abbreviations: cAMP, cyclic adenosine monophosphate; CAP, catabolite activator protein.

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KAKO POVEĆATI PRINOS INDUCIBILNIH ENZIMA KOD MIKROORGANIZAMA

Živomir Petronijević

U radu su navedene metode za povećanje prinosa, pre svega, inducibilnih enzima. Jedan broj metoda je zasnovan na optimizovanju uslova kultivacije odabranog producenta, pre svega na promeni sastava hranljive podloge; između ostalog primenom induktora i isključenjem sastojaka koji izazivaju kataboličku represiju ili represiju konačnim proizvodom. Druga grupa metoda su one koje zu bazirane na dobijanju i selekciji mutanata, kako onih konstitutivnih i otpornih na kataboličku represiju, tako i onih kod kojih do povećanja prinosa dolazi preko drugih mehanizama.