



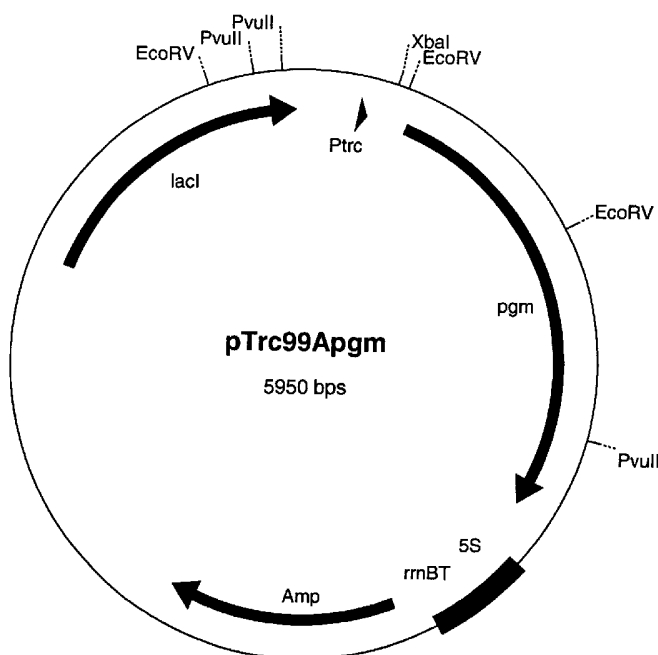
(10) International Publication Number
WO 02/101055 A2

PCT

- Published:**
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/101055 A2



(57) Abstract: The invention relates to a process for the preparation of D-pantothenic acid and/or salts thereof or feedstuffs additives comprising these by fermentation of microorganisms of the Enterobacteriaceae family, in particular those which already produce D-pantothenic acid, in which the nucleotide sequence(s) in the microorganisms which code(s) for the pgm gene is/are enhanced, in particular over-expressed.

**Process for the Preparation of D-Pantothenic Acid
and/or Salts Thereof**

Field of the Invention

This invention relates to a process for the preparation of
5 D-pantothenic acid and salts thereof or mixtures comprising
these using microorganisms of the Enterobacteriaceae family
in which at least the *pgm* gene is enhanced.

Prior Art

Pantothenic acid is produced worldwide in an order of
10 magnitude of several thousand tons a year. It is used inter
alia in human medicine, in the pharmaceuticals industry and
in the foodstuffs industry. A large portion of the
pantothenic acid produced is used for nutrition of stock
animals such as poultry and pigs.

15 Pantothenic acid can be prepared by chemical synthesis, or
biotechnologically by fermentation of suitable
microorganisms in suitable nutrient solutions. In the
chemical synthesis, DL-pantolactone is an important
precursor. It is prepared in a multi-stage process from
20 formaldehyde, isobutylaldehyde and cyanide, and in further
process steps, the racemic mixture is separated, D-
pantolactone is subjected to a condensation reaction with
 β -alanine, and D-pantothenic acid is obtained in this way.

The typical commercial form is the calcium salt of D-
25 pantothenic acid. The calcium salt of the racemic mixture
of D,L-pantothenic acid is also customary.

The advantage of the fermentative preparation by
microorganisms lies in the direct formation of the desired
stereoisomeric form, that is to say the D-form, which is
30 free from L-pantothenic acid.

Various types of bacteria, such as e.g. *Escherichia coli* (E. coli), *Arthrobacter ureafaciens*, *Corynebacterium erythrogenes*, *Brevibacterium ammoniagenes*, and also yeasts, such as e.g. *Debaromyces castellii*, can produce D-

5 pantothenic acid in a nutrient solution which comprises glucose, DL-pantoic acid and β -alanine, as shown in EP-A 0 493 060. EP-A 0 493 060 furthermore shows that in the case of E. coli, the formation of D-pantothenic acid is improved by amplification of pantothenic acid biosynthesis

10 genes from E. coli which are contained on the plasmids pFV3 and pFV5 in a nutrient solution comprising glucose, DL-pantoic acid and β -alanine.

EP-A 0 590 857 and US Patent 5,518,906 describe mutants derived from E. coli strain IFO3547, such as FV5714, FV525,

15 FV814, FV521, FV221, FV6051 and FV5069, which carry resistances to various antimetabolites, such as salicylic acid, α -ketobutyric acid, β -hydroxyaspartic acid, O-methylthreonine and α -ketoisovaleric acid. They produce pantoic acid in a nutrient solution comprising glucose, and

20 D-pantothenic acid in a nutrient solution comprising glucose and β -alanine. It is furthermore stated in EP-A 0 590 857 and US Patent 5,518,906 that after amplification of the pantothenic acid biosynthesis genes panB, panC and panD, which are said to be contained on the plasmid pFV31,

25 in the above-mentioned strains the production of D-pantoic acid in nutrient solutions comprising glucose and the production of D-pantothenic acid in a nutrient solution comprising glucose and β -alanine is improved.

WO97/10340 furthermore reports on the favorable effect of

30 the enhancement of the *ilvGM* operon on the production of D-pantothenic acid. Finally, EP-A-1001027 reports on the effect of the enhancement of the *panE* gene on the formation of D-pantothenic acid.

According to the prior art, the D-pantothenic acid or the

35 corresponding salt is isolated from the fermentation broth

and purified (EP-A-0590857 and WO96/33283) and used accordingly in purified form, or the fermentation broth comprising D-pantothenic acid is dried in total (EP-A-1050219) and used in particular as a feedstuffs additive.

5 Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of D-pantothenic acid and/or salts thereof, and animal feedstuffs additives comprising these.

10 Summary of the Invention

When D-pantothenic acid or pantothenic acid or pantothenate are mentioned in the following text, this means not only the free acids but also the salts of D-pantothenic acid, such as e.g. the calcium, sodium, ammonium or potassium
15 salt.

The invention provides a process for the preparation of D-pantothenic acid and/or salts thereof using microorganisms of the Enterobacteriaceae family which in particular already produce D-pantothenic acid and in which at least
20 one, preferably endogenous nucleotide sequence(s) which code(s) for the pgm gene is enhanced, in particular over-expressed.

"Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences
25 present in the population of a species.

In particular, the process is a process which is characterized in that the following steps are carried out:

- a) fermentation of microorganisms of the Enterobacteriaceae family which produce D-pantothenic acid and in which at least the pgm
30 gene is enhanced, in particular over-expressed;

the gene which codes for phosphoglucomutase and optionally alleles of this gene are enhanced, in particular over-expressed, under conditions suitable for the formation of the gene product;

5 further genes of the pantothenic acid biosynthesis pathway are optionally attenuated or enhanced at the same time in order to increase the production of pantothenic acid;

10 b) the fermentation is optionally carried out in the presence of alkaline earth metal compounds, these being added to the fermentation broth continuously or discontinuously in preferably stoichiometric amounts;

15 c) concentration of the D-pantothenic acid or the corresponding salts in the medium or the fermentation broth or optionally in the cells of the microorganisms of the Enterobacteriaceae family; and

20 d) after conclusion of the fermentation, isolation of the D-pantothenic acid, and/or of the corresponding salt(s).

The invention also provides a process in which, after conclusion of the fermentation, some or all (≥ 0 to 100 %) of the biomass remains in the fermentation broth, and the
25 broth obtained in this way is processed, optionally after concentration, to a solid mixture which comprises D-pantothenic acid and/or salts thereof and preferably comprises further constituents from the fermentation broth.

These further constituents are, above all, the dissolved
30 compounds which originate from the feed medium and soluble organic compounds which are formed.

Detailed Description of the Invention

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, of the ORF (Open Reading Frame) or ORFs, using a potent promoter or a gene or allele or ORF which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding enzyme or protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or wild-type enzyme or the activity or concentration of the protein or enzyme in the starting microorganism.

The microorganisms which the present invention provides can produce D-pantothenic acid from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They are representatives of Enterobacteriaceae, in particular of the genus *Escherichia*. Of the genus *Escherichia*, the specie *Escherichia coli* is to be mentioned in particular. Within the species *Escherichia coli* the so-called K-12 strains, such as e. g. the strains MG1655 or W3110 (Neidhard et al.: *Escherichia coli* and *Salmonella*. Cellular and Molecular Biology (ASM Press, Washington D.C.)) or the *Escherichia coli* wild type strain IFO3547 (Institute of Fermentation, Osaka, Japan) and mutants derived from these which have the ability to produce D-pantothenic acid are suitable.

Suitable D-pantothenic acid-producing strains of the genus *Escherichia*, in particular of the species *Escherichia coli*, are, for example

Escherichia coli FV5069/pFV31
Escherichia coli FV5069/pFV202
Escherichia coli FE6/pFE80 and
Escherichia coli KE3

- 5 It has been found that Enterobacteriaceae produce D-pantothenic acid in an improved manner after enhancement, in particular over-expression of the pgm gene. The use of endogenous genes is preferred.

The nucleotide sequences of the genes or open reading
10 frames (ORF) of Escherichia coli belong to the prior art and can also be found in the genome sequence of Escherichia coli published by Blattner et al. (Science 277, 1453 - 1462 (1997)).

The following information, inter alia, on the pgm gene can
15 be found in the prior art:

Description: Phosphoglucomutase

Alternative gene name: pgmU

EC No.: 5.4.2.1

Reference: Lu and Kleckner, Journal of Bacteriology
20 176:5847-5851 (1994)

Accession No.: AE000172

The gene described in the text reference stated can be used according to the invention. Alleles of the gene or open reading frames which result from the degeneracy of the
25 genetic code or due to sense mutations of neutral function can furthermore be used, the activity of the proteins being substantially unchanged.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and
30 regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is

additionally possible to increase the expression in the course of fermentative D-pantothenic acid production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is
5 also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome.
Alternatively, an over-expression of the genes in question
10 can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Chang and Cohen (Journal of Bacteriology 134:1141-1156 (1978)), in Hartley and Gregori (Gene 13:347-
15 353 (1981)), in Amann and Brosius (Gene 40:183-190 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80:21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11, 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26:222-224
20 (1991)), in Quandt and Klipp (Gene 80:161-169 (1989)), in Hamilton (Journal of Bacteriology 171:4617-4622 (1989)), in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998) and in known textbooks of genetics and molecular biology.

25 Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102, 75-78 (1991)), pTrc99A (Amann et al.; (Gene 69:301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National
30 Academy of Science USA 80 (21):6557-6561 (1983)) can be used. A strain transformed with one or more plasmid vectors where the plasmid vector(s) carries at least one nucleotide sequence which codes for the pgm gene can be employed in a process according to the invention.

It may furthermore be advantageous for the production of D-pantothenic acid with strains of the Enterobacteriaceae family, in addition to the enhancement of the pgm gene, for one or more of the genes chosen from the group consisting
5 of

- the ilvGM operon which codes for acetohydroxy-acid synthase II (WO 97/10340),
- the panB gene which codes for ketopantoate hydroxymethyl transferase (US-A-5,518,906),
- 10 • the panE gene which codes for ketopantoate reductase (EP-A-1001027),
- the panD gene which codes for aspartate decarboxylase (US-A-5,518,906),
- the panC gene which codes for pantothenate synthetase
15 (US-A-5,518,906),
- the glyA gene which codes for serine hydroxymethyl transferase (Plamann et al., Nucleic Acids Research 11(7):2065 -2075(1983)),
- the genes gcvT, gcvH and gcvP which code for the glycine
20 cleavage system (Okamura-Ikeda et al., European Journal of Biochemistry 216, 539-548 (1993)),
- the serA gene which codes for phosphoglyceric acid dehydrogenase (Tobey und Grant, Journal of Biological Chemistry 261:12179-12183(1986)),
- 25 • the serA(FBR) allele which codes for "feed back" resistant variants of phosphoglyceric acid dehydrogenase (DE-A-4232468),
- the serC gene which codes for phosphoserine transaminase (Duncan und Coggins, Biochemical Journal 234:49-57
30 (1986)),

- the bfr gene which codes for bacterioferrin (Andrews et al., Journal of Bacteriology 171:3940-3947 (1989)),
- the hns gene which codes for the DNA-binding protein HLP-II (reference: Pon et al., Molecular and General
5 Genetics 212:199-202 (1988)),
- the mdh gene which codes for malate dehydrogenase (Sutherland und McAlister-Henn, Journal of Bacteriology 1985 163:1074-1079 (1985)),
- the cysK gene which codes for cysteine synthase A
10 (Boronat et al., Journal of General Microbiology 130:673-685 (1984)),
- the fda gene which codes for fructose bisphosphate aldolase (class II) (Alefounder et al., Biochemical Journal 257:529-534 (1989)),
- 15 • the dldH gene which codes for NADH-dependent lipoamide dehydrogenase (reference: Stephens et al., European Journal of Biochemistry 135:519-527 (1983)),
- the pepB gene which codes for peptidase B (Hermsdorf et al., International Journal of Peptide and Protein
20 Research 13:146-151 (1979); Suzuki et al., Journal of Fermentation and Bioengineering 82:392-397 (1996); Suzuki et al., Journal of Bacteriology 183(4):1489-1490, (2001)),
- the aldH gene which codes for NADP-dependent aldehyde
25 dehydrogenase (Heim and Strehler, Gene 99:15-23 (1991)) and
- the adk gene which codes for adenylate kinase (Brune et al., Nucleic Acids Research 13:7139-7151 (1985))

to be enhanced, in particular over-expressed, individually
30 or together. The use of endogenous genes is preferred.

Finally, it may be advantageous for the production of D-pantothenic acid with strains of the Enterobacteriaceae family, in addition to the enhancement of the pgm gene, for one or more of the genes chosen from the group consisting
5 of

- the avtA gene which codes for transaminase C (EP-A-1001027)
- the poxB gene which codes for pyruvate oxidase (Grabau and Cronan, Nucleic Acids Research. 14 (13), 5449-5460
10 (1986))
- the pckA gene which codes for PEP carboxykinase (Medina et al., Journal of Bacteriology 172, 7151-7156 (1990))

to be attenuated, in particular eliminated or expressed at a low level, individually or together.

15 The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a
20 corresponding enzyme or protein with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

By attenuation measures, including reduction in expression, the activity or concentration of the corresponding protein
25 is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

In addition to over-expression of the pgm gene it may
30 furthermore be advantageous for the production of D-pantothenic acid to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing

Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982). Bacteria in which the metabolic pathways which reduce the formation of D-pantothenic acid are at least
5 partly eliminated can be employed in the process according to the invention.

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch (feed process) or the repeated fed batch process
10 (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart,
15 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions
20 of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981). Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose,
25 molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as
30 the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds,
35 such as ammonium sulfate, ammonium chloride, ammonium

phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or
5 dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth
10 substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Precursors of pantothenic acid, such as aspartate, β -alanine, ketoisovalerate, ketopantoic acid or pantoic acid and optionally salts thereof, can moreover be added to the
15 culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds,
20 such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.

For the preparation of alkaline earth metal salts of pantothenic acid, in particular the calcium salt or magnesium salt, it is equally possible to add the
25 suspension or solution of an inorganic compound containing an alkaline earth metal, such as, for example, calcium hydroxide or MgO, or of an organic compound, such as the alkaline earth metal salt of an organic acid, for example calcium acetate, continuously or discontinuously during the
30 fermentation. For this purpose, the cation necessary for preparation of the desired alkaline earth metal salt of D-pantothenic acid is introduced into the fermentation broth directly in the desired amount, preferably in an amount of 0.95 to 1.1 equivalents.

However, the salts can also be formed after conclusion of the fermentation by addition of the inorganic or organic compounds to the fermentation broth, from which the biomass has optionally been removed beforehand.

5 Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-
10 containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. The pH is in general between 5.0 to 8.0, preferably 5.5 to 7.6. The fermentation is continued until a maximum of D-
15 pantothenic acid has formed. This target is usually reached within 10 hours to 160 hours.

The D-pantothenic acid or the corresponding salts of D-pantothenic acid contained in the fermentation broth can then be isolated and purified in accordance with the prior
20 art.

It is also possible for the fermentation broths comprising D-pantothenic acid and/or salts thereof preferably first to be freed from all or some of the biomass by known separation methods, such as, for example, centrifugation,
25 filtration, decanting or a combination thereof. However, it is also possible to leave the biomass in its entirety in the fermentation broth. In general, the suspension or solution is preferably concentrated and then worked up to a powder, for example with the aid of a spray dryer or a
30 freeze-drying unit. This powder is then in general converted by suitable compacting or granulating processes, e. g. also build-up granulation, into a coarser-grained, free-flowing, storable and largely dust-free product with a particle size distribution of preferably 20 to 2000 µm, in
35 particular 100 to 1400µm. In the granulation or compacting

it is advantageous to employ conventional organic or inorganic auxiliary substances or carriers, such as starch, gelatin, cellulose derivatives or similar substances, such as are conventionally used as binders, gelling agents or
5 thickeners in foodstuffs or feedstuffs processing, or further substances, such as, for example, silicas, silicates or stearates.

Alternatively, the fermentation product, with or without further of the conventional fermentation constituents, can
10 be absorbed, in particular sprayed, on to an organic or inorganic carrier substance which is known and conventional in feedstuffs processing, such as, for example, silicas, silicates, grits, brans, meals, starches, sugars or others, and/or stabilized with conventional thickeners or binders.
15 Use examples and processes in this context are described in the literature (Die Mühle + Mischfüttertechnik 132 (1995) 49, page 817).

These mixtures comprising the carrier substances can also be processed to a product with the desired particle size
20 distribution by granulation processes.

D-Pantothenic acid and/or the desired salt of D-pantothenic acid or a formulation comprising these compounds is optionally added in a suitable process stage during or after the fermentation in order to achieve or establish the
25 content of pantothenic acid desired in the product or the desired salt.

The desired content of pantothenic acid and/or the desired salt is in general in the range from 20 to 80 wt.% (based on the dry weight).

30 The concentration of pantothenic acid can be determined with known chemical (Velisek; Chromatographic Science 60, 515-560 (1992)) or microbiological methods, such as e.g.

the Lactobacillus plantarum test (DIFCO MANUAL, 10th Edition, p. 1100-1102; Michigan, USA).

The present invention is explained in more detail in the following with the aid of embodiment examples.

- 5 The minimal (M9) and complete media (LB) for Escherichia coli used are described by J.H. Miller (A Short Course in Bacterial Genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, ligation, Klenow and
10 alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular cloning - A laboratory manual, (1989), Cold Spring Harbor Laboratory Press). The transformation of Escherichia coli is carried out by the method of Chung et al. (Proceedings of the National Academy
15 of Sciences of the United States of America (1989) 86: 2172-2175) or by the method of Chuang et. al. (Nucleic Acids Research (1995) 23: 1641).

Example 1

Construction of the expression plasmid pTrc99Apgm

- 20 The pgm gene from E. coli K12 is amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the pgm gene in E. coli K12 MG1655 (Accession Number AE000172, Blattner et al. (Science 277, 1453 - 1462
25 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany).

Primer pgm5': 5' - CGTTGCAGACAAAGGACAAAGC - 3'
(SEQ ID No. 1)

- Primer pgm3': 5' - GCGACCGCCCTTTTTTTATTAAATGTG - 3'
30 (SEQ ID No. 2)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 1700 bp in size can be amplified
5 with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer's instructions with
10 the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10. Selection of plasmid-carrying cells takes place on LB agar, to which 50 µg/ml kanamycin are added. After isolation of the
15 plasmid DNA, the vector pCR-Blunt II-TOPO-pgm is cleaved with the restriction enzymes SpeI and XbaI and, after separation in 0.8% agarose gel, the pgm fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Amersham
20 Biosciences, Freiburg, Germany) is cleaved with the enzyme XbaI, subsequently dephosphorylated with alkaline phosphatase according to the manufacturer's instructions (Amersham Biosciences, Freiburg, Germany) and ligated with the pgm fragment isolated. The E. coli strain XL1-Blue MRF'
25 (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin is added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes EcoRV and PvuII. The
30 plasmid is called pTrc99Apgm (Figure 1).

Example 2

Preparation of the strains FE6-1/pTrc99A and FE6-1/pTrc99Apgm

The E. coli strain FE6 is a valine-resistant mutant of E.
35 coli K12 MG1655 (US-B-6171845) and is deposited as DSM12379

at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Starting from FE6, after incubation at 37°C on minimal agar, to which
5 2 g/L glucose and 1 g/L β -hydroxyaspartic acid are added, spontaneous mutants are isolated. A selected β -hydroxyaspartic acid-resistant individual colony is then incubated on minimal agar, which comprises 2 g/L glucose and 0.2 g/L O-methylthreonine, at 37°C. After this step, a
10 mutant called FE6-1 is resistant to L-valines, α -ketoisovaleric acid, β -hydroxyaspartic acid and O-methylthreonine. A pure culture of the strain FE6-1 was deposited on 8th September 2000 as DSM13721 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ =
15 German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

The plasmids pTrc99A and pTrc99Apgm are transformed individually into the strain FE6-1 and plasmid-carrying cells are selected on LB agar, to which 50 μ g/ml ampicillin
20 are added. The strains obtained are called FE6-1/pTrc99A and FE6-1/pTrc99Apgm.

Example 3

Preparation of D-pantothenic acid with strains derived from FE6-1

25 The pantothenate production of the E. coli strains FE6-1/pTrc99A and FE6-1/pTrc99Apgm is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(\text{NH}_4)_2\text{SO}_4$, 1 g/l KH_2PO_4 , 0,5 g/l
30 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g/l CaCO_3 , 20 g/l glucose, 50 mg/l ampicillin are inoculated with an individual colony and incubated for 20 hours at 33°C and 200 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland). In each case 200 μ l of this preculture are transinoculated into 10 ml of

production medium (25 g/l $(\text{NH}_4)_2\text{SO}_4$, 2 g/l KH_2PO_4 , 1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.018 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 30 g/l CaCO_3 , 20 g/l glucose, 20 g/l β -alanine, 250 mg/l thiamine) and the batch is incubated for 48 hours at 37°C. After the
5 incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of the D-pantothenate formed is then
10 determined in the culture supernatant centrifuged off by means of High Performance Liquid Chromatography [column: Reversed Phase MZ-Aqua Perfect (diameter 4,6 mm), mobile Phase 25 mM acetate buffer with 10 % methanol, flow rate 1 ml/min, RI detector].

15 The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	Pantothenate mg/l
FE6-1/pTrc99A	8.7	47
FE6-1/pTrc99Apgm	8.9	57

Brief Description of the Figure:

- Figure 1: Map of the plasmid pTrc99Apgm containing the
5 pgm gene.

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- Amp: Ampicillin resistance gene
- 10 • lacI: Gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- pgm: Coding region of the pgm gene
- 5S: 5S rRNA region
- 15 • rrnBT: rRNA terminator region
- bps Base pairs

The abbreviations for the restriction enzymes have the following meaning:

- SpeI: Restriction endonuclease from *Sphaerotilus* species
20 ATCC 13923

- XbaI: Restriction endonuclease from *Xanthomonas campestris*
- EcoRV: Restriction endonuclease from *Escherichia coli* B946
- 5 • PvuII: Restriction endonuclease from *Proteus vulgaris*

What is claimed is:

1. Process for the preparation of D-pantothenic acid and salts thereof or feedstuffs additives comprising this/these compound(s) by fermentation of
5 microorganisms of the Enterobacteriaceae family, in particular those which already produce D-pantothenic acid, in which at least the nucleotide sequence(s) which code(s) for the pgm gene is/are enhanced, in particular over-expressed, under conditions suitable
10 for the formation of the pgm gene product phosphoglucomutase.
2. Process according to claim 1, wherein microorganisms transformed with plasmids which carry the pgm gene are employed.
- 15 3. Process according to claim 2, wherein transformed microorganisms in which the pgm gene is integrated into the chromosome are employed.
4. Process according to claim 1, wherein microorganisms in which the promoter and regulation region upstream of
20 the structural gene is mutated to achieve the over-expression are employed.
5. Process according to claim 1, wherein microorganisms in which expression cassettes are incorporated upstream of the structural gene to achieve the over-expression are
25 employed.
6. Process according to claim 1, wherein to achieve the enhancement, the life of the mRNA read off as the matrix from the above-mentioned sequences is prolonged and/or the breakdown of the corresponding enzyme
30 protein(s) is prevented.
7. Process according to claims 1 to 6, wherein microorganisms which have additional metabolite or

antimetabolite resistance mutations, individually or together, are employed.

8. Process according to claims 1 to 7, wherein to achieve the over-expression the microorganisms are fermented in an appropriately modified culture medium or the fermentation procedure is modified.
9. Process according to claim 1, in which
 - a) the D-pantothenic acid and/or salts thereof are concentrated in the fermentation broth or in the cells of the microorganisms, and
 - b) after the end of the fermentation the desired product(s) is/are isolated, the biomass and/or further constituents of the fermentation broth being separated off in an amount of ≥ 0 to 100 %.
10. Process according to claim 1, wherein the microorganisms of the Enterobacteriaceae family belong to the genus Escherichia.
11. Process according to claim 1, wherein the microorganisms originate from the genus Escherichia, in particular the species Escherichia coli.
12. Process according to claim 1, wherein one or more of the preferably endogenous genes chosen from the group consisting of the following is or are additionally enhanced, in particular over-expressed:
 - 12.1 the ilvGM operon which codes for acetohydroxy-acid synthase II,
 - 12.2 the panB gene which codes for ketopantoate hydroxymethyl transferase,
 - 12.3 the panE gene which codes for ketopantoate reductase,

- 12.4 the panD gene which codes for aspartate decarboxylase,
- 12.5 the panC gene which codes for pantothenate synthetase,
- 5 12.6 individually or together, the genes gcvT, gcvH and gcvP which code for the glycine cleavage system
- 12.7 the glyA gene which codes for serine hydroxymethyl transferase,
- 10 12.8 the serA gene which codes for phosphoglyceric acid dehydrogenase,
- 12.9 the serA(FBR) allele which codes for "feed back" resistant variants of phosphoglyceric acid dehydrogenase,
- 15 12.10 the serC gene which codes for phosphoserine transaminase,
- 12.11 the bfr gene which codes for bacterioferrin,
- 12.12 the hns gene which codes for the DNA-binding protein HLP-II,
- 20 12.13 the mdh gene which codes for malate dehydrogenase,
- 12.14 the cysK gene which codes for cysteine synthase A,
- 12.15 the fda gene which codes for fructose bisphosphate aldolase (class II),
- 25 12.16 the dldH gene which codes for NADH-dependent lipoamide dehydrogenase,
- 12.17 the pepB gene which codes for peptidase,

- 12.18 the aldH gene which codes for NADP-dependent aldehyde dehydrogenase,
- 12.19 the adk gene which codes for adenylate kinase.
13. Process according to claim 1, wherein microorganisms are employed in which genes which code for the metabolic pathways which reduce the formation of D-pantothenic acid are at least partly eliminated, in particular the
- 13.1 avtA gene which codes for transaminase C, and
- 13.2 the pckA gene which codes for PEP carboxykinase.
14. Process according to claim 1, wherein the expression of the polynucleotide(s) which code(s) for the poxB gene is attenuated, in particular eliminated.
15. Process for the preparation of feedstuffs additives comprising D-pantothenic acid and/or salts thereof, according to claim 1, wherein
- a) from a fermentation broth obtained by fermentation of the microorganisms and comprising D-pantothenic acid and/or salts thereof, the biomass and/or a portion of the constituents are separated off in an amount of ≥ 0 to 100%,
- b) the mixture obtained in this way is optionally concentrated (concentration),
- c) the resulting mixture comprising the pantothenic acid and/or the pantothenate is converted into a finely divided powder by known measures, and
- d) a free-flowing animal feedstuffs additive with a particle size distribution of 20 to 2000 μm , in

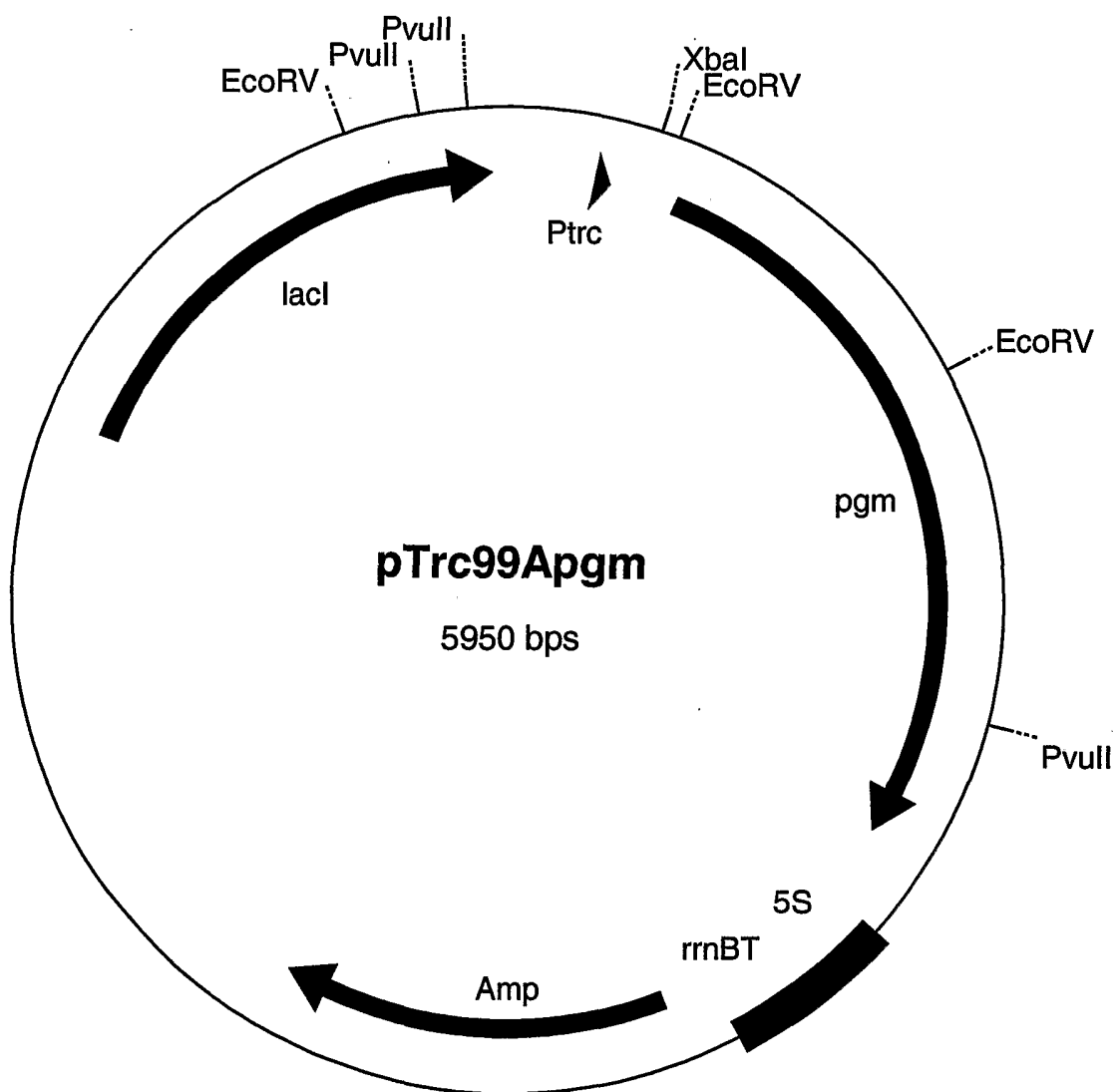
particular 100 to 1400 μm , is obtained therefrom by suitable measures.

16. Process according to claim 15 with a content of D-pantothenic acid and salts thereof or salts thereof, chosen from the group consisting of the magnesium or calcium salt, from fermentation broths, wherein the fermentation is carried out in the presence of compounds of Ca or Mg, these being fed in continuously or discontinuously in stoichiometric amounts in particular, and a product comprising a Ca or Mg salt of D-pantothenic acid or consisting thereof is obtained.
17. Process according to claim 15 with a content of D-pantothenic acid and salts thereof or salts thereof, chosen from the group consisting of the magnesium or calcium salt, from fermentation broths, wherein after the fermentation compounds of calcium or magnesium are added to the fermentation broth, in particular in stoichiometric amounts, optionally after separating off ≥ 0 to 100% of the biomass formed.
18. Process according to claim 15, wherein before or after the concentration, D-pantothenic acid or one or more of the pantothenic acid salts mentioned is/are added to the fermentation broth, the amount of compounds added being such that the total concentration thereof in the animal feedstuffs additive is in the range from 20 to 80 wt.% (dry weight).
19. Process according to claims 15 to 18, wherein an animal feedstuffs additive with the desired particle size is obtained from the fermentation broth, optionally after addition of D-pantothenic acid and/or salts thereof and optionally after addition of organic or inorganic auxiliaries, by
- a) drying and compacting, or

- b) spray drying, or
- c) spray drying and granulation, or
- d) spray drying and build-up granulation.

20. Process according to claim 15, wherein the fermentation
5 broth is applied to an inorganic auxiliary, in
 particular to a silica or silicate, optionally after
 removal of the biomass (≥ 0 to 100%).
21. Microorganisms of the Enterobacteriaceae family which
10 produce pantothenic acid and in which the pgm gene is
 present in enhanced form.

Figure 1:



SEQUENCE PROTOCOL

5 <110> Degussa AG

 <120> Process for the preparation of D-pantothenic acid and/or
 salts thereof

10 <130> 020019 BT

 <160> 2

 <170> PatentIn version 3.1

15 <210> 1

 <211> 22

 <212> DNA

 <213> artificial sequence

20 <220>

 <221> misc_feature

 <222> (1)..(22)

 <223> Primer pgm5'

25 <400> 1

 cgttgcagac aaaggacaaa gc 22

 <210> 2

 <211> 27

30 <212> DNA

 <213> artificial sequence

 <220>

 <221> misc_feature

35 <222> (1)..(27)

 <223> Primer pgm3'

 <400> 2

 gcgaccgccc tttttttatt aaatgtg 27

40