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(54) Title: STABILIZED ENZYME COMPOSITIONS

(57) Abstract: The present invention relates to a composition comprising an enzyme and a transition metal ion. Additionally, the present invention relates to a composition comprising a long-chain alcohol.



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STABILIZED ENZYME COMPOSITIONS

Field of the invention

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The present invention relates to a composition comprising an enzyme and a transition metal ion. Additionally, the present invention relates to a composition comprising a long-chain alcohol.

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Background of the invention

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The complex chemical structure of enzymes, displaying many different functional groups, not only gives enzymes their unprecedented specificity and reactivity in catalyzing a wide range of conversions, but also is the reason that enzymes are relatively labile compounds. Clearly this phenomenon is the origin of the fact that studies for optimizing enzyme stability are continuously ongoing resulting in a multitude of often specific solutions to a general problem.

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Enzymes may be destabilized by unfolding of the three-dimensional structure of the enzyme or by chemical degradation. De-stabilization can easily occur from contact with polar solvents, microbial attack, electrolytes, surfactants, temperature and extreme pH. In order to compensate loss of enzyme activity during periods of storage, formulators may use excess enzymes in liquid enzymatic compositions. However, this is an unfavorable solution as enzymes are relatively expensive formulation ingredients. This problem may be overcome by adding stabilizers. Materials that have been used for stabilizing enzymes include various organic and inorganic compounds such as polyols, carboxylic acids, carboxylic acid salts, carboxylic acid esters, and sugars; calcium salts; boron compounds, and various combinations thereof. Protein extracts can also be used to stabilize enzymes through inhibition of the enzyme.

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Nevertheless, due to the wide variety of enzymes alternative solutions to the problem of enzyme de-stabilization are still required and will be required in the future.

Detailed description of the invention

The combination of an enzyme and a metal is known. As a matter of fact, a certain class of enzymes, *i.e.* the metalloenzymes, can only function by virtue of the presence of a metal. Metalloenzyme is a generic term for an enzyme that contains a metal ion cofactor. Indeed, about one quarter to one third of all enzymes require metals to carry out their functions. The metal ion is usually coordinated by nitrogen, oxygen or sulfur atoms belonging to amino acids in the polypeptide chain and/or a macrocyclic ligand incorporated into the enzyme. The presence of the metal ion allows metalloenzymes to perform functions such as redox reactions that cannot easily be performed by the limited set of functional groups found in amino acids (A. Messerschmidt *et al.*, (2001) Handbook of Metalloproteins; Wiley, ISBN 0-471-62743-7). Usually the amount of these metals, such as transition metals, is quite low such that the concentration in formulations does not exceed 1-100 $\mu\text{mol/kg}$. As a matter of fact, higher concentrations are quite often toxic to the enzyme.

In the context of the present invention, the term transition metal (sometimes also called a transition element) refers to an element whose atom has an incomplete d sub-shell, or which can give rise to cations with an incomplete d sub-shell. This definition corresponds to groups 3 to 11 on the periodic table.

In a first aspect the present invention provides a composition comprising an enzyme and a transition metal ion. It has surprisingly been found that certain, relatively high, concentrations of transition metal have a stabilizing effect on enzymes. Thus, in a composition comprising an enzyme, a concentration of transition metal ion ranging from 2 mmol/kg to 100 mmol/kg leads to enhanced enzyme stability. Preferably said transition metal is present in a concentration ranging from 2.5 mmol/kg to 50 mmol/kg, more preferably from 3 mmol/kg to 25 mmol/kg. Preferably the transition metal of the present invention is cobalt or manganese.

In one embodiment of the first aspect of the present invention, the enzyme is a hydantoin racemase. Polypeptides with hydantoin racemase activity, also called hydantoin racemases, are known in the art. They have been found in a variety of organisms, for instance WO 01/23582 describes a hydantoin racemase from *Arthrobacter aurescens* (DSM 3747) and JP 04271784 describes a hydantoin racemase from *Pseudomonas* NS 672 (Watabe *et al.*, J. Bact. **174**, 3461-3466 (1992)). Hydantoin

racemase have also been described in *Sinorhizobium meliloti* (acc. nr. CAC 47181, Capela *et al.*, Proc. Natl. Acad. Sci. **98**, 9877-9882 (2001)), in *Microbacterium liquefaciens* (acc. nr. CAD 32593, EP 1188826), and in *Agrobacterium tumefaciens* strain C58 (acc. nrs. AAL 45498, AAK 88746 and AAK 90298, Las Heras-Vazquez *et al.*, Biochem. Biophys. Res. Commun. **303**, 541-547 (2003), Wood *et al.*, Science **294**, 2317-2323 (2001) and Hinkle *et al.*, NCBI database, Complete Genome Sequence of *Agrobacterium tumefaciens* C58 (*Rhizobium radiobacter* C58), the Causative Agent of Crown Gall Disease in Plants. Direct Submission, submitted August 14, 2001). Isolated polypeptides that exhibit hydantoin racemase and that are void of substrate inhibition have been described in WO 2003/100050. Not unusually hydantoin racemase implies the presence of more than one enzyme such as a hydantoinase and a racemase. It has been found that the present invention also applies to mixtures comprising additional enzymes such carbamoylases.

In a second embodiment the present invention provides a composition comprising an enzyme, a transition metal and an alcohol. Preferred alcohols are long-chain alcohols such as the hexanols, the heptanols, the octanols, the nonanols and the like. Specifically well-functioning alcohols are 1-heptanol, 1-octanol and 1-nonanol. The concentration wherein said alcohol is present in the composition preferably is in an amount of from 0.1% to 5% by weight of the total composition. It has been established that the presence of alcohol enhances the stabilizing effect of the transition metal.

In a second aspect of the present invention there is provided a method for the preparation of a composition comprising an enzyme and a transition metal ion comprising the steps of contacting said enzyme with a salt of said transition metal. Said contacting may be carried out during production of said enzyme, for instance during a fermentation process. The transition metal salt may be added as such to a solution comprising the enzyme or to the process mixture wherein the enzyme is produced or in an aqueous solution. Suitable salts of transition metals are halides, sulfates, carbonates, phosphates, nitrates and the like. Examples are cobalt bromide, cobalt chloride, cobalt nitrate, cobalt sulfate, manganese bromide, manganese chloride, manganese nitrate, manganese sulfate and the like.

In one embodiment the enzyme is produced in a fermentation process and the resulting mixture is the composition of the first aspect of the invention. The mixture may

be concentrated, for instance by evaporation, diafiltration, lyophilization, microfiltration, ultrafiltration and similar or other techniques known to the skilled person.

Legend to the Figure

Figure 1 shows the influence of the presence of manganese (Mn^{2+}) and octanol on the residual activity over time of L-hydantoinase from *Escherichia coli* RV308. The Y-axis represents the residual activity in % relative to the activity at start which is set at 100%. The X-axis represents the time (h) of incubation. Legend: $\diamond$ = blank (no added Mn^{2+} or octanol); Δ = 1 mM Mn^{2+} ; $\blacktriangle$ = 5 mM Mn^{2+} ; $\blacklozenge$ = 10 mM Mn^{2+} ; $\circ$ = octanol; $\bullet$ = octanol + 1 mM Mn^{2+} ; $\blacksquare$ = octanol + 5 mM Mn^{2+} ; $\square$ = octanol + 10 mM Mn^{2+} . It can be seen that addition of 1 mM, 5 mM and 10 mM Mn^{2+} results in an increase of residual activity compared to the blank sample. Combination with the addition of octanol, which in itself also has an effect on stabilizing the enzyme activity, further enhances the positive contribution of manganese.

EXAMPLES

Materials and methods

Unless indicated otherwise, all molecular techniques employed were essentially performed according to Maniatis *et al.* (J. Sambrook, E.F. Fritsch, T. Maniatis. Molecular Cloning 2nd edition. CSH Press).

Protocol for transformation of pKECaroP-hyu1 construct into *Escherichia coli* RV308

- Thaw *Escherichia coli* RV308 aliquots (200 μ l, super competent) on ice
- Add 15 μ l LR reaction mix (see above)
- Incubate 30 minutes on ice
- Heat shock 1 minute 42°C
- Cool cells 2 minutes on ice
- Add 1 ml LB medium (5 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone)
- Incubate 1 hour 37°C

- Plate on LB agar plates supplemented with kanamycine (5 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone, 15 g/l agar, 50 mg/l kanamycine)
- Incubate 24 hours 28°C
- Isolate single colonies

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Protocol for expression of Hyu genes in Escherichia coli RV308

Single clones from the transformation (see above) were used to inoculate 5 ml of 2xTY media (10g/l yeast extract, 16g/l tryptone, 5g/l NaCl) supplemented with 0.05g/l kanamycine and 1 mM MnCl₂ or CoCl₂, respectively. The culture was incubated at 28°C and 150 rpm for 24 hours and then used for inoculation of 100 ml 2xTY media supplemented with 0.05 g/l kanamycine and 1 mM MnCl₂ or CoCl₂, respectively. The cultures were again incubated for 24–28 hours under conditions previously mentioned and subsequently harvested by centrifugation (20 minutes, 5000 rpm, 4°C). The cell pellet was resuspended in 5 ml Tris-HCl (100 mM, pH 7), centrifuged again (20 minutes, 5000 rpm, 4°C) and the cells were frozen at –20°C.

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Analysis methods

Hydantoinase activity assay

Unit definition: One unit of hydantoinase activity is defined as the amount of enzyme producing 1 µmol of N-carbamoyl phenylalanine per minute at pH 8.0 and 40°C.

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Substrate: 100 mM D/L-phenylalanine hydantoin suspension in 130 mM TRIS/HCl buffer pH 8.0 also containing 1.43 mM MnCl₂.

Sample pre-treatment: One gram of sample is suspended in 10 mL 130 mM TRIS/HCl buffer pH 8.0 also containing 1.43 mM MnCl₂. After mixing, the suspension is diluted to approximately 0.9 U/mL with the same buffer. Samples are kept on ice before use. The linear range of this method is from 0.16 to 1.62 U/mL

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Assay: 2.1 mL substrate suspension is brought in a reaction tube and subsequently pre-heated for 10 minutes in a 40°C water bath. The reaction is started by adding 100 µL of sample and mixing. A substrate blank is included by incubating the substrate with 100 µL buffer instead of sample. After 30 minutes the enzymatic reaction is stopped by adding 400 µL 1 M HCl solution followed by mixing and subsequent cooling in ice water. The reaction mixture is filtered over a 0.45 µm filter. The clear solution is transferred into a HPLC injection vial.

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Standards: 1 mM N-carbamoyl-L-phenylalanine and L-phenylalanine.

HPLC analysis of reaction mixture and standards:

- Column: Xbridge® phenyl 5 µm, 4.6 X 150 mm, Waters
- Detector: UV@220nm
- Flow rate: 1.2 mL/min
- 5 • Injection volume: 20 µl
- Sample tray temp.: 10°C
- Column temp.: ambient
- Run time: 20 minutes
- Mobile phase A: 40 mM phosphate buffer; pH 5.2/Acetonitrile (98/2 (v/v))
- 10 • Mobile phase B: 40 mM phosphate buffer; pH 5.2/Acetonitrile (70/30 (v/v))
- Gradient:

Time [min]	Mobile phase A [%]	Mobile phase B [%]
0	100	0
13	0	100
15	100	0
20	100	0

Retention times (may differ depending on the HPLC system used): 3.40 minutes:

L-phenylalanine; 5.17 minutes: N-carbamoyl-L-phenylalanine; 9.96 minutes: substrate phenylalanine-hydantoin.

Calculation: The response factors for 1 mM of the standards N-carbamoyl-L-phenylalanine and L-phenylalanine are calculated using the following formulas:

$$RF_{N-cpa} = \frac{\text{Peak area}_{N-cpa} \times MW_{N-cpa} \times V_k \times Df_{N-cpa} \times 100}{W_{N-cpa} \times P_{N-cpa} \times 1000}$$

$$RF_{Phe} = \frac{\text{Peak area}_{Phe} \times MW_{Phe} \times V_k \times Df_{Phe} \times 100}{W_{Phe} \times P_{Phe} \times 1000}$$

Where:

RF_{N-cpa} = Response Factor of 1 mM N-carbamoyl-phenylalanine [mAU.min.L/mmol]

RF_{Phe} = Response Factor of 1 mM phenylalanine [mAU.min.L/mmol]

Peak area_{N-cpa} = Peak area N-carbamoyl-phenylalanine [mAU.min]

Peak area_{Phe} = Peak area phenylalanine [mAU.min]

V_k = Flask volume of standard solution [mL]

Df_{N-cpa} = Total dilution factor of N-carbamoyl-phenylalanine standard solution [mL]

Df_{Phe} = Total dilution factor of phenylalanine standard solution [mL]

W_{N-cpa} = Weight of N-carbamoyl-phenylalanine [mg]

W_{Phe} = Weight of phenylalanine [mg]

P_{N-cpa} = Purity of N-carbamoyl-phenylalanine [%]

5 P_{Phe} = Purity of phenylalanine [%]

MW_{N-cpa} = Molecular weight N-carbamoyl-phenylalanine (208 g/mol)

MW_{Phe} = Molecular weight phenylalanine (165.19 g/mol)

The hydantoinase activity is calculated using the following formula:

U/g =

$$\left\{ \left(\frac{\text{Area Sample}_{N-cpa} - \text{Area blank}_{N-cpa}}{RF_{N-cpa}} \right) + \left(\frac{\text{Area Sample}_{Phe} - \text{Area blank}_{Phe}}{RF_{Phe}} \right) \right\} \times \left(\frac{Df_{sam} \times V_k \times V_t}{V_{sam} \times t \times W} \right)$$

10 Where:

$\text{Area Sample}_{N-cpa}$ = Peak area of N-carbamoyl-phenylalanine of sample

$\text{Area blank}_{N-cpa}$ = Peak area of N-carbamoyl-phenylalanine of blank

Area Sample_{Phe} = Peak area of phenylalanine of sample

Area blank_{Phe} = Peak area of phenylalanine of blank

15 V_t = Total reaction volume (mL)

Df_{sam} = Dilution factor sample

V_{sam} = Volume sample (mL)

V_k = Flask volume of sample

t = Time of incubation (min)

20 W = Weight sample (g)

Carbamoylase activity assay

Unit definition: One unit of carbamoylase activity is defined as the amount of enzyme producing 1 μmol of phenylalanine per minute at pH 8.0 and 40°C.

25 Substrate: 100 mM N-carbamoyl-L-phenylalanine suspension in 130 mM TRIS/HCl buffer pH 8.0 also containing 1.43 mM MnCl_2 .

Sample pre-treatment: One gram of sample is suspended in 10 mL 130 mM TRIS/HCl buffer pH 8.0 also containing 1.43 mM MnCl_2 . After mixing, the suspension is diluted to approximately 1.5 U/mL with the same buffer. Samples are kept on ice before use.

30 The linear range of this activity assay is from 0.32 to 3.15 U/mL.

Assay: See hydantoinase assay.

Standards: 1 mM L-phenylalanine.

HLPC analysis of reaction mixture and standard: See hydantoinase assay

Calculation: The response factor for the 1 mM L-phenylalanine standard is calculated using the following formula:

$$RF_{Phe} = \frac{\text{Peak area}_{Phe} \times MW_{Phe} \times V_k \times Df_{Phe} \times 100}{W_{Phe} \times P_{Phe} \times 1000}$$

5 Where:

RF_{Phe} = Response Factor of 1 mM phenylalanine [mAU x min x L/mmol]

Peak area_{Phe} = Peak area phenylalanine [mAU x min]

V_k = Flask volume of phenylalanine standard solution [mL]

Df_{Phe} = Dilution factor of phenylalanine standard solution

10 W_{Phe} = Weight of phenylalanine [mg]

P_{Phe} = Purity of phenylalanine [%]

MW_{Phe} = Molecular weight phenylalanine [165.19 mg/mmol]

The carbamoylase activity is calculated using the following formula:

$$U/g = \left(\frac{\text{Area Sample}_{Phe} - \text{Area blank}_{Phe}}{RF_{Phe}} \right) \times \left(\frac{Df_{sam} \times V_k \times V_t}{V_{sam} \times t \times W} \right)$$

15 Where:

Area Sample_{Phe} = Peak area of phenylalanine of sample [mAU x min]

Area blank_{Phe} = Peak area of phenylalanine of blank [mAU x min]

V_t = Total reaction volume [mL]

Df_{sam} = Dilution factor sample

20 V_k = Flask volume of sample

V_{sam} = Volume sample [mL]

t = Time of incubation [min]

W = Weight sample [g]

25 Racemase activity assay

Unit definition: One unit of racemase activity is defined as the amount of enzyme producing 1 μmol of L-phenylalanine-hydantoin from D-phenylalanine-hydantoin per minute at pH 8.0 and 37°C.

30 Substrate: 10 mM D-phenylalanine-hydantoin solution in 130 mM TRIS/HCl buffer pH 8.0 also containing 0.1 M EDTA. Solution must be made at 37°C.

Sample pre-treatment: One gram of sample is suspended in 10 mL 130 mM TRIS/HCl buffer pH 8.0 also containing 0.1 M EDTA. After mixing, the suspension is diluted to

approximately 0.5 U/mL with the same buffer. Samples are kept on ice before use. Linear range of the assay is from 0.19 to 1.16 U/mL.

Assay: 2.0 mL pre-heated substrate solution is brought in a reaction tube in a 37°C water bath. After 2 minutes the reaction is started by adding 100 µL of sample and mixing. A substrate blank is included by incubating the substrate with 100 µL buffer instead of sample. After 30 minutes the enzymatic reaction is stopped by adding 400 µL 1 M NaOH solution followed by mixing. The reaction mixture is filtered over a 0.45 µm filter. The clear solution is transferred into a HPLC injection vial.

Standards: 1 mM L-phenylalanine-hydantoin and 1 mM N-carbamoyl-L-phenylalanine

HLPC analysis of reaction mixture and standard:

- Column, w. precolumn: Chirobiotic T (250 mm x 4.6 mm I.D., 5 µm), Astec
- Detector: UV@220nm
- Flow rate: 1.5 mL/min
- Injection volume: 20 µl
- Sample tray temp.: 10°C
- Column temp.: ambient
- Run time: 8 minutes, isocratic
- Mobile phase: 15 mM ammonium acetate pH 4.1/20% Methanol

Retention times (may differ depending on the HPLC system used): 5.46 minutes:

substrate D-phenylalanine-hydantoin; 7.21 minutes: product L-phenylalanine-hydantoin.

When hydantoinase is not completely inhibited by EDTA, then peaks of L- and D-carbamoyl-phenylalanine can be visible at approx. 2.8 and 3.5 minutes, respectively.

Calculation

The response factor for the 1 mM L-phenylalanine standard is calculated using the following formula:

$$RF_{LPH} = \frac{\text{Peak area}_{LPH} \times MW_{LPH} \times V_{k_{LPH}} \times 100}{W_{LPH} \times P_{LPH} \times 1000}$$

Where:

RF_{LPH} = Response Factor of 1 mM L-phenylalanine-hydantoin

Peak area_{LPH} = Peak area L-phenylalanine-hydantoin [mAU x min]

$V_{k_{LPH}}$ = Flask vol. of L-phenylalanine-hydantoin standard solution [mL]

W_{LPH} = Weight of L-phenylalanine-hydantoin [mg]

P_{LPH} = Purity of L-phenylalanine-hydantoin [%]

MW_{LPH} = Molecular weight L-phenylalanine-hydantoin [190 g/mol]

The response factor for 1mM of the standard N-carbamoyl-L-phenylalanine is calculated using the following formula:

$$RF_{LCP} = \frac{\text{Peak area}_{LCP} \times MW_{LCP} \times V_{k_{LCP}} \times 100}{W_{LCP} \times P_{LCP} \times 1000}$$

Where:

- 5 RF_{LCP} = Response Factor of 1 mM N-carbamoyl-L-phenylalanine
 Peak area_{LCP} = Peak area N-carbamoyl-L-phenylalanine [mAU x min]
 $V_{k_{LCP}}$ = Flask vol. of N-carbamoyl-L-phenylalanine standard [mL]
 W_{LCP} = Weight of N-carbamoyl-L-phenylalanine [mg]
 P_{LCP} = Purity of N-carbamoyl-L-phenylalanine [%]
 10 MW_{LCP} = Molecular weight N-carbamoyl-L-phenylalanine [208 g/mol]

The racemase activity is calculated using the following formula:

$$U/g = \left\{ \left(\frac{\text{Area Sample}_{LPH} - \text{Area blank}_{LPH}}{RF_{LPH}} \right) + \left(\frac{\text{Area Sample}_{LCP} - \text{Area blank}_{LCP}}{RF_{LCP}} \right) \right\} \times \left(\frac{Df_{sam} \times V_k \times V_t}{V_{sam} \times t \times W} \right)$$

Where:

- Area Sample_{LPH} = Peak area of L-phenylalanine-hydantoin of sample [mAU x min]
 15 Area blank_{LPH} = Corr. area of L-phenylalanine-hydantoin of blank [mAU x min]
 Area Sample_{LCP} = Area of N-carbamoyl-L-phenylalanine of sample [mAU x min]
 Area blank_{LCP} = Peak area of N-carbamoyl-L-phenylalanine of blank [mAU x min]
 V_t = Total reaction volume [mL]
 Df_{sam} = Dilution factor sample
 20 V_{sam} = Volume sample [mL]
 t = Time of incubation [min]
 V_k = Flask volume of sample [mL]
 W = Weight sample [g]

- 25 The corrected peak area of L-phenylalanine-hydantoin of the blank is necessary to correct for the spontaneous racemisation that occurs during the time the samples are in the HPLC and is calculated as follows. The difference of the blanks at the end of the series and start of the series is divided by number of runs between them. This value represents the increase in LPH during each run. This value is added to the value of the first blank, multiplied by the amount of runs between the sample and the first blank.

Example 1

Construction of a clone for co-expression of L-hydantoinase, L-carbamoylase and hydantoin racemase in *Escherichia coli* RV308

5 The aim was to obtain active coexpression of the L-hydantoinase from *Arthrobacter aureus* (HyuH), the L-carbamoylase from *Bacillus stearothermophilus* (HyuC) and the hydantoin racemase from *Agrobacterium radiobacter* (HyuA) in the host *Escherichia coli* RV308 resulting in a production strain for the production of L-amino acids. The sequences of the 3 enzymes are known from the following literature sources:

- 10
- L-hydantoinase from Abendrodt *et al.* Biochemistry 41 (27), 8589-8597 (2002);
 - L-carbamoylase from Battise *et al.* Appl. Environ. Microbiol. 63(2), 763-766 (1997);
 - Hydantoin racemase from EP 1506294 B1.

An operon was synthetically prepared according to WO 2008/067981 wherein the three genes of the hydantoin pathway (hyuH, hyuC, hyuA) are separated from each other by
15 spacers containing a ribosomal binding site rbs (Shine-Delgarno Sequence) and a restriction site for further subcloning. The DNA sequences of the enzyme-encoding regions were optimized for expression in *Escherichia coli* RV308.

The Hyu1 operon was subsequently cloned into an expression vector. The expression vector pKECaro_hyu1 is derived from plasmid pKECtrp (described in WO 00/66751) by
20 replacing the trp promoter ==> PenG acylase expression cassette by the aroH promoter ==> hyu1 operon. The DNA was transformed into supercompetent *Escherichia coli* RV308 cells (as described in Material and Methods) and single clones were isolated from the agar plate. The clones were grown in LB medium supplemented with kanamycin (5 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone, 50 mg/l kanamycin) and
25 plasmid DNA was isolated using the Qiagen Miniprep Kit (following the standard procedure). The accuracy of the constructs was checked by restriction analysis.

Example 2

Fermentation of in *Escherichia coli* RV308 expressing L-hydantoinase, L-carbamoylase and hydantoin racemase activity

30 Transformed supercompetent *Escherichia coli* RV308 cells as described in Example 1 were fermented at pH 7.15±0.15 and 27.0±0.5°C using the fermentation medium outlined

in Table 1 wherein glucose and thiamine were fed during the process. The pH was controlled with NH_3 (25%). At the end of the fermentation (approx. 100 h), 1-octanol (4.0 g/kg) and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (2.4 g/kg) were added after which the broth was cooled to $\leq 5 \pm 1^\circ\text{C}$.

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Table 1 Composition of fermentation medium

Component	Concentration (g/kg)
Yeast extract	24.6
Citric acid. H_2O	10.0
K_2HPO_4	8.9
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.20
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	3.1
CaCl_2 as 25% solution	4.6
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.51
$(\text{NH}_4)_2\text{SO}_4$	5.0
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.006
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	0.004
H_3BO_3	0.004
Basildon (antifoam)	0.15
Glucose	10.5
Neomycin sulfate	0.10
Thiamine.HCl	0.014

Example 3

10 **Stability L-hydantoinase, L-carbamoylase and hydantoin racemase at 4°C with and without addition of manganese and octanol**

A sample from the fermentation broth obtained in Example 2 was used for stability testing for the enzymes L-hydantoinase, L-carbamoylase and hydantoin racemase in the absence and presence of Mn^{2+} and/or octanol at three different incubation times. The results are summarized in the below overview.

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Sample	Incubation time (h)	Hydantoinase (U/mL)	Carbamoylase (U/mL)	Racemase (U/mL)
Series 1	4	580	18	26
Series 1	25	123	12	18
Series 1	48	74	6.3	16
Series 2: + Mn ²⁺	4	619	18	20
Series 2: + Mn ²⁺	25	303	16	26
Series 2: + Mn ²⁺	48	258	16	26
Series 3: + octanol + Mn ²⁺	4	3840	20	23
Series 3: + octanol + Mn ²⁺	25	3776	20	26
Series 3: + octanol + Mn ²⁺	48	3645	18	24

Example 4

Stability L-hydantoinase at 4°C with and without addition of manganese and octanol; end of fermentation with 1 mM Mn²⁺

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A sample from the fermentation broth obtained in Example 2 was used for stability testing for L-hydantoinase in the absence and presence of 1 mM Mn²⁺ and/or octanol at five different incubation times. The results are summarized in the below overview.

Addition	Residual activity (%)				
	0 h	3 h	24 h	46 h	144 h
None	100	45	58	57	47
1mM Mn ²⁺	100	54	77	72	57
5mM Mn ²⁺	100	59	81	78	74
10mM Mn ²⁺	100	56	80	71	58
Octanol	100	68	103	113	98
Octanol+ 1mM Mn ²⁺	100	76	124	142	130
Octanol+ 5mM Mn ²⁺	100	57	126	149	148
Octanol + 10mM Mn ²⁺	100	52	128	151	149

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Example 5

Stability L-hydantoinase at 4°C with and without addition of manganese and octanol; end of fermentation with 3 mM Mn²⁺

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A sample from the fermentation broth obtained in Example 2 was used for stability testing for L-hydantoinase in the absence and presence of 3 mM Mn^{2+} and/or octanol at five different incubation times. The results are summarized in the below overview.

Addition	Residual activity (%)				
	0 h	3 h	24 h	46 h	144 h
None	100	48	70	74	50
1mM Mn^{2+}	100	56	86	82	57
5mM Mn^{2+}	100	74	86	89	62
10mM Mn^{2+}	100	60	83	85	58
Octanol	100	94	117	126	110
Octanol+ 1mM Mn^{2+}	100	90	134	145	138
Octanol+ 5mM Mn^{2+}	100	51	102	162	164
Octanol + 10mM Mn^{2+}	100	50	112	158	165

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Example 6

Multilevel factorial design analysis on the stability of L-hydantoinase, L-carbamoylase and hydantoin racemase vs variations in time, temperature and presence or absence of manganese, octanol and flocculant

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A sample from the fermentation broth obtained in Example 2 was used for multilevel factorial design analysis on the stability of L-hydantoinase, L carbamoylase and hydantoin racemase vs variations in time, temperature and presence or absence of Mn^{2+} , octanol and flocculant. The results are summarized in Table 2.

15	Column A:	Random order	Column G:	Manganese (mM)
	Column B:	Standard order	Column H:	Flocculant (g/L)
	Column C:	Mixture number	Column I:	Hydantoinase (U/g)
	Column D:	Time (days)	Column J:	Carbamoylase (U/g)
	Column E:	Temperature (°C)	Column K:	Hydantoin racemase (U/g)
20	Column F:	Octanol (g/L)		

After running a Pareto chart from the above experiments it can be concluded that:

- For hydantoinase addition of octanol and Mn^{2+} has a strong positive initial effect, there is a strong positive effect from the interaction between temperature and Mn^{2+} and there are strong negative effects from interaction of octanol/flocculant and Mn^{2+} /flocculant. The stability in the presence of octanol/ Mn^{2+} at 4°C is good.

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- For carbamoylase there is a strong positive effect by addition of Mn^{2+} and there is a strong negative effect from the flocculant. The stability in the presence of octanol/ Mn^{2+} at 4°C is good.

For hydantoin racemase there is a strong negative effect from the flocculant and the stability in the presence of octanol/ Mn^{2+} at 4°C is good.

Table 2 Multilevel factorial design analysis on the stability of L-hydantoinase

A	B	C	D	E	F	G	H	I	J	K
33	1	1	0	4	0	0	0	334	12.5	19.3
39	2	1	2	4	0	0	0	140	10.7	18.8
31	3	1	4	4	0	0	0	174	12.0	18.8
32	4	1	0	20	0	0	0	339	13.4	19.1
8	5	1	2	20	0	0	0	192	15.4	18.5
11	6	1	4	20	0	0	0	229	15.9	18.2
6	7	5	0	4	4	0	0	560	14.8	18.8
28	8	5	2	4	4	0	0	404	12.7	17.6
30	9	5	4	4	4	0	0	386	14.0	15.4
35	10	5	0	20	4	0	0	628	14.8	18.4
38	11	5	2	20	4	0	0	461	13.1	19.6
29	12	5	4	20	4	0	0	561	11.5	13.1
40	13	3	0	4	0	10	0	365	16.5	18.8
24	14	3	2	4	0	10	0	244	16.6	18.3
14	15	3	4	4	0	10	0	342	17.7	17.9
44	16	3	0	20	0	10	0	371	16.1	18.2
41	17	3	2	20	0	10	0	449	19.2	18.4
16	18	3	4	20	0	10	0	517	19.7	18.0
27	19	7	0	4	4	10	0	2530	18.6	18.7
37	20	7	2	4	4	10	0	2220	18.2	17.3
42	21	7	4	4	4	10	0	2220	18.2	18.0
9	22	7	0	20	4	10	0	2450	18.7	20.1
13	23	7	2	20	4	10	0	2350	15.1	15.2
36	24	7	4	20	4	10	0	2400	13.2	14.6
7	25	2	0	4	0	0	1	589	13.2	15.1
3	26	2	2	4	0	0	1	391	13.4	14.1
45	27	2	4	4	0	0	1	478	11.8	14.5
23	28	2	0	20	0	0	1	678	10.5	14.6
22	29	2	2	20	0	0	1	336	12.5	14.4
4	30	2	4	20	0	0	1	461	13.6	14.5
1	31	6	0	4	4	0	1	463	13.8	10.5
47	32	6	2	4	4	0	1	427	11.3	11.6
21	33	6	4	4	4	0	1	528	11.4	11.9
46	34	6	0	20	4	0	1	427	12.0	12.9
19	35	6	2	20	4	0	1	549	10.2	11.9
2	36	6	4	20	4	0	1	569	8.4	12.1
18	37	4	0	4	0	10	1	1240	15.4	16.1
5	38	4	2	4	0	10	1	832	15.5	15.3
26	39	4	4	4	0	10	1	660	14.6	15.0
25	40	4	0	20	0	10	1	1160	14.3	15.5
12	41	4	2	20	0	10	1	692	15.6	17.4
43	42	4	4	20	0	10	1	929	15.5	14.3
10	43	8	0	4	4	10	1	1200	17.2	11.6
20	44	8	2	4	4	10	1	1140	13.6	14.0
15	45	8	4	4	4	10	1	1260	12.1	15.7
34	46	8	0	20	4	10	1	1300	16.1	11.7
17	47	8	2	20	4	10	1	1740	10.0	12.8
48	48	8	4	20	4	10	1	1720	8.9	8.3
Ref								304	12.4	17.3

CLAIMS

1. Composition comprising an enzyme and a transition metal ion, characterized in that the concentration of said transition metal ion is from 2 mmol/kg to 100 mmol/kg.
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2. A composition according to claim 1 wherein said transition metal is cobalt or manganese.
3. A composition according to any one of claims 1 to 2 wherein said enzyme is a
10 hydantoinase.
4. A composition according to claim 3 further comprising a carbamoylase.
5. A composition according to any one of claims 3 to 4 further comprising a
15 racemase.
6. A composition according to any one of claims 1 to 5 further comprising an alcohol.
- 20 7. A composition according to claim 6 wherein said alcohol is 1-octanol.
8. A composition according to any one of claims 6 to 7 wherein said alcohol is present in an amount of from 0.1% to 5% by weight of the total composition.
- 25 9. Method for the production of a composition comprising an enzyme and 2 mmol/kg to 100 mmol/kg of a transition metal ion, wherein said enzyme is contacted with a salt of said transition metal.

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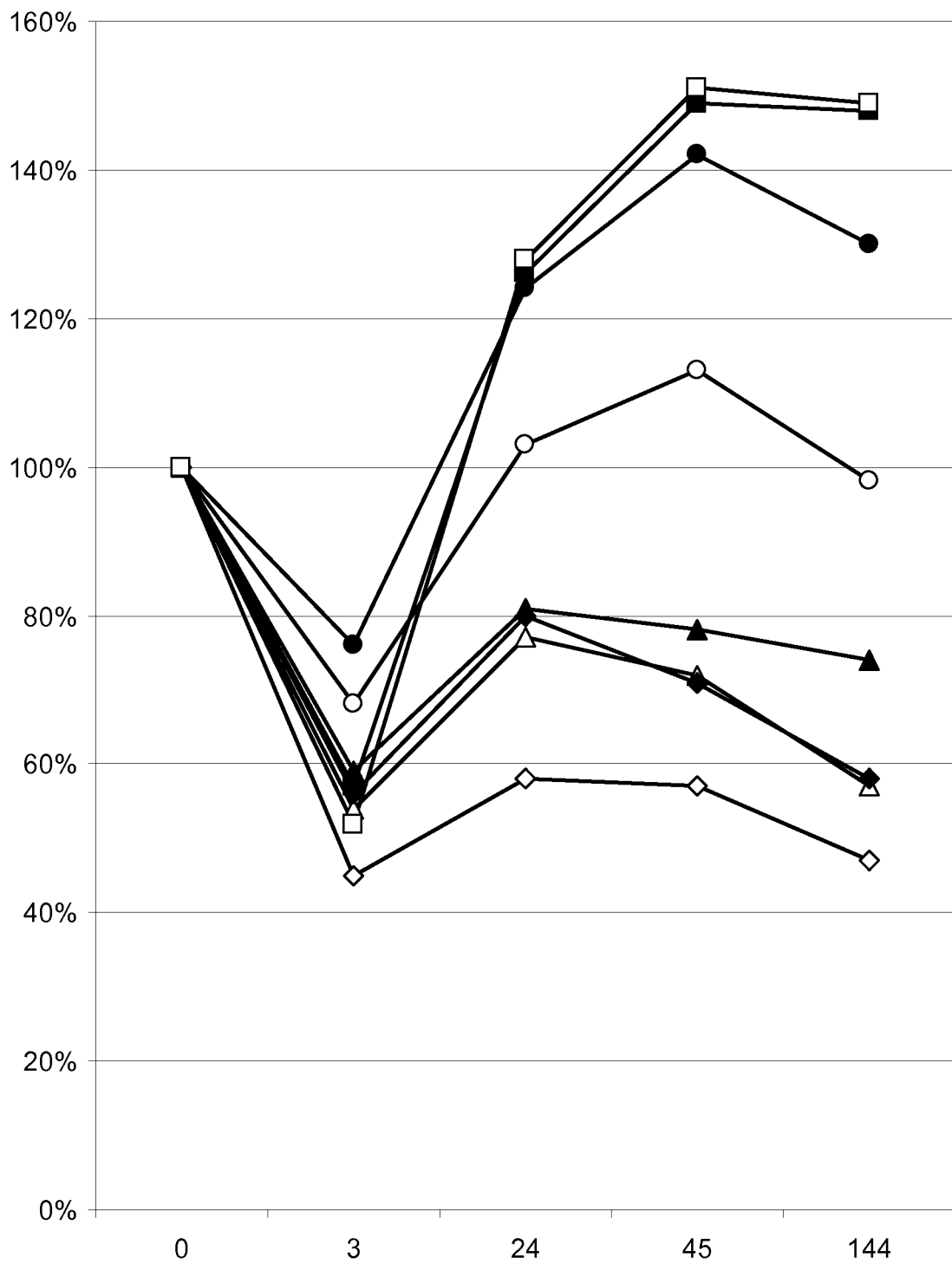


Figure 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/058406

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/86 C12N9/96
ADD.

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)
C12N

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

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

EPO-Internal, BIOSIS, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 6 030772 A (NIPPON SODA CO) 8 February 1994 (1994-02-08)	1-6,9
A	the whole document	7,8
X	WO 02/081626 A2 (SQUIBB BRISTOL MYERS CO [US]; POLITINO MICHAEL [US]; TONZI SEAN M [US]) 17 October 2002 (2002-10-17)	1-6,9
A	page 37 - page 38	7,8
X	EP 0 739 978 A2 (GRACE W R & CO [US]) 30 October 1996 (1996-10-30)	1-6,9
A	page 6 - page 7	7,8
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A	page 2; claims 1-10	7,8
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search

12 August 2010

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18/08/2010

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/058406

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

International application No

PCT/EP2010/058406

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