Processes for the production of hydroxycinnamic acids using polypeptides having tyrosine ammonia lyase activity.

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(54) **Title**: PROCESSES FOR THE PRODUCTION OF HYDROXYCINNAMIC ACIDS USING POLYPEPTIDES HAVING TYROSINE AMMONIA LYASE ACTIVITY

(57) **Abstract**: The present invention generally relates to the field of biotechnology as it applies to the production of hydroxycinnamic acids using polypeptides having tyrosine ammonia lyase activity. More particularly, the present invention pertains to polypeptides having tyrosine ammonia lyase activity and high substrate specificity towards tyrosine, which makes them particularly suitable in the production of p-coumaric acid and other hydroxycinnamic acids. The present invention thus provides processes for the production of p-coumaric acid and other hydroxycinnamic acids employing these polypeptides as well as recombinant host cells expressing same.

Processes for the production of hydroxycinnamic acids using polypeptides having tyrosine ammonia lyase activity

Field of the invention

The present invention generally relates to the field of biotechnology as it applies to the production of hydroxycinnamic acids using polypeptides having tyrosine ammonia lyase activity. More particularly, the present invention pertains to polypeptides having high tyrosine ammonia lyase activity and high substrate specificity towards tyrosine, which makes them particularly suitable for the production of p-coumaric acid and other hydroxycinnamic acids. The present invention thus provides processes for the production of p-coumaric acid and other hydroxycinnamic acids employing these polypeptides as well as recombinant host cells expressing same.

Background of the invention

Small organic molecules of interest to the biotech industry often involve aromatic structures that are derived from p-coumaric acid (pHCA) or other hydroxycinnamic acids. In particular, pHCA is a precursor for many secondary metabolites including flavonoids and stilbenes, and has a significant potential as a building block for producing polymers. pHCA is naturally formed from phenylalanine by subsequent ammonialyase and hydroxylase reactions or directly from tyrosine by the deamination of tyrosine.

Aromatic amino acid lyases constitute an enzymatic family, and are classified by their substrate specificity as being histidine ammonia-lyases (HAL, EC 4.3.1.3), tyrosine ammonia-lyases (TAL, EC 4.3.1.23), phenylalanine ammonia-lyases (PAL, EC 4.3.1.24) or phenylalanine/tyrosine ammonia-lyases (PAL/TAL, EC 4.3.1.25). Enzymes categorized as acting on either of the structurally similar amino acids tyrosine or phenylalanine are normally having some activity towards the other (Rosler et al.,1997; Zhu et al.,2013). Similar enzymatic families are tyrosine 2,3-aminomutases (TAM, EC 5.4.3.6) and phenylalanine aminomutase (PAM, EC 5.4.3.11) (Christenson et al.,2003a; Jin et al.,2006). All of these proteins contain a prosthetic group, 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO) formed by the cyclization of the sequential three amino acids alanine, serine and glycine. TAMs as well as PAMs have been shown to have background lyase activity (Christenson et al.,2003b; Walker et al.,2004). The lyase and mutase activities of a single enzyme may be subject to a thermal switch (Chesters et al.,2012), and mutations can divert the enzymatic

activity of a PAM into higher PAL activity (Bartsch et al.,2013). Aminomutases have been found in the biosynthetic pathways to antitumor drug compounds.

A number of tyrosine ammonia lyases have been cloned and functionally characterized: While PAL and TAL activities had been shown in plant extracts previously, Kyndt et al (Kyndt *et al.*,2002) identified and characterized the first TAL enzyme, originating from the purple non-sulfur bacterium Rhodobacter capsulatus, which uses pHCA as a chromophore in the light-sensing photoactive yellow protein (PYP). The actinomycete *Saccharothrix espanaensis* produce two related oligosaccharide antibiotics saccharomicin A and B, both containing a substructure derived from pHCA, which is formed by the *sam8* gene of the antibiotic biosynthetic gene cluster (Berner *et al.*,2006; Strobel *et al.*,2012). EncP is a PAL playing a role in the biosynthetic pathway to enterocin in *Streptomyces maritimus* (Xiang; Moore,2002), and recently, another TAL was identified in an actinomycete, namely *bagA* in *Streptomyces* sp. Tü 4128 (Zhu *et al.*,2012), and as a part of biosynthetic route to bagremycin A and B. *stlA* of Photorhabdus luminescens is also part of an antibiotic biosynthetic pathway, yet StlA has PAL activity (Williams *et al.*,2005). A number of the TALs have been purified and enzymatically characterized (Appert *et al.*,1994; Rosler *et al.*,1997; Kyndt *et al.*,2002; Christenson *et al.*,2003b; Williams *et al.*,2005; Berner *et al.*,2006; Schroeder *et al.*,2008; Bartsch; Bornscheuer,2009).

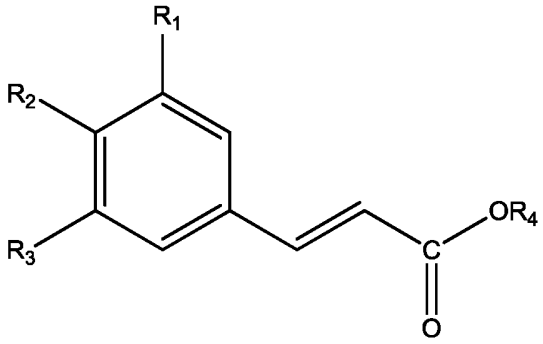
TAL enzymatic activity has been described in patent literature and in particular the enzymes of the yeast genus *Rhodotorula*, the yeasts Phanerochaete chrysosporium and *Trichosporon cutaneum*, and the purple non-sulfur bacteria Rhodobacter sphaeroides and capsulatus. However, since these enzymes also show some specificity towards phenylalanine, they are not particularly useful in the production of p-coumaric acid and other hydroxycinnamic acids due to accompanying contamination by cinnamic acid as a result of the deamination of phenylalanine.

Accordingly, there is a need in the art for biological processes which allow the production of p-coumaric acid and other hydroxycinnamic acids at high yield and high purity. This need is solved by the present invention.

Summary of the invention

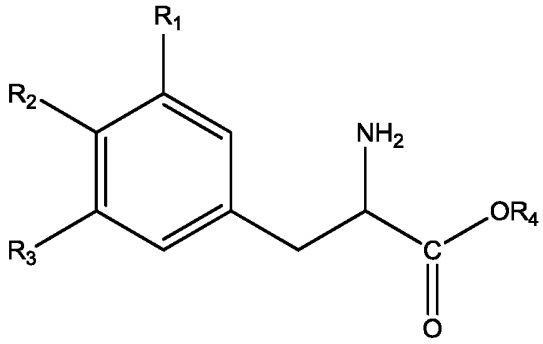
The present invention is based on the identification of enzymes of bacterial origin, which show higher TAL activity compared to previously characterized enzymes. The identified enzymes show improved specificity and productivity, and thus allow the enhanced biologically production of hydroxycinnamic acids such as pHCA.

The present invention thus provides in a first aspect a method for producing a hydroxycinnamic acid of general formula I



general formula I

, the method comprises deaminating a compound of general formula II



general formula II

wherein R_1, R_2 and R_3 independently are selected from the group consisting of hydrogen (H), hydroxyl (-OH), C_{1-6}-alkyl and C_{1-6}-Alkoxy, provided that at least one of R_1, R_2 and R_3 is hydroxyl (-OH); and R_4 is selected from the group consisting of hydrogen (-H) and C_{1-6}-alkyl;

using a polypeptide as detailed herein. Particularly, the method involves the use of a polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g. SEQ ID NO: 1);

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g. SEQ ID NO: 1); or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g. SEQ ID NO: 1), wherein 1 to 50, such as 1 to 40, 1 to 35, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 1 to 3, amino acid residues are substituted, deleted, and/or inserted.

The present invention provides in a further aspect a recombinant host cell comprising a polypeptide as detailed herein. Particularly, the recombinant host cell according to the present invention comprises a heterologous polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g. SEQ ID NO: 1);

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g. SEQ ID NO: 1); or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g. SEQ ID NO: 1), wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted.

The present invention provides in yet a further aspect a method for producing a hydroxycinnamic acid of general formula I comprising the step of contacting a recombinant host cell as detailed herein with a medium comprising a compound of the general formula II. The method may further comprise the step culturing the recombinant host cell under suitable conditions for the production of the hydroxycinnamic acid, and further optionally the recovery of the hydroxycinnamic acid.

The present invention provides in yet a further aspect the use of a polypeptide as detailed herein in the production of a hydroxycinnamic acid of general formula I, and particularly in the production of p-coumaric acid.

Brief description of the drawings

Figure 1: Map of plasmid for expression of FjXAL in *E. coli*

Figure 2: Map of plasmid for expression of HaXAL1 in *E. coli*

Figure 3: Map of plasmid for expression of HaXAL2 in *E. coli*

Figure 4: Map of plasmid for expression of His-tagged FjXAL in *E. coli*

Figure 5: Map of plamid for expression of FjXAL in *S. cerevisiae*

Figure 6: Map of plamid for expression of FjXAL in *S. cerevisiae*

Figure 7: Map of plamid for expression of HaXAL1 in *S. cerevisiae*

Figure 8: Map of plamid for expression of HaXAL1 in *S. cerevisiae*

Figure 9: Specific p-coumaric acid (pHCA) and cinnamic acid (CA) productivities of strains expressing TAL/PAL enzymes in CDM

Detailed description of the invention

Unless specifically defined herein, all technical and scientific terms used have the same meaning as commonly understood by a skilled artisan in the fields of biochemistry, genetics, and molecular biology.

All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will prevail. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Current Protocols in Molecular Biology (Frederick M. AUSUBEL, 2000, Wiley and son Inc, Library of Congress, USA); Molecular Cloning: A Laboratory Manual, Third Edition, (Sambrook et al, 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Harries & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the series, Methods In ENZYMOLOGY (J. Abelson and M. Simon, eds.-in-chief, Academic Press, Inc., New York), specifically, Vols.154 and 155 (Wu et al. eds.) and Vol. 185, "Gene Expression Technology" (D. Goeddel, ed.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Immunochemical

Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); and Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Polypeptides and host cells

As indicated above, the present invention provides and utilizes polypeptides having tyrosine ammonia lyase activity and high substrate specificity towards tyrosine. This makes them particularly suitable for the production of p-coumaric acid and other hydroxycinnamic acids.

Particularly, the polypeptides employed according to the invention are polypeptides selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1);

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1); or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1), wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted.

According to certain embodiments, a polypeptide according to the invention is a polypeptide according to i). Accordingly, a polypeptide according to the invention may be a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1). According to particular embodiments, a polypeptide according to i) comprises an amino acid sequence set forth in SEQ ID NO: 1. According other particular embodiments, a polypeptide according to i) comprises an amino acid sequence set forth in SEQ ID NO: 2. According to yet other particular embodiments, a polypeptide according to i) comprises an amino acid sequence set forth in SEQ ID NO: 3.

According to other certain embodiments, a polypeptide according to the invention is a polypeptide according to ii). Accordingly, a polypeptide according to the invention may be a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1). According to particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 80%, such as at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1). According to other particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 85%, such as at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1). According to other particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1). According to other particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1).

According to particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1. According to more particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 80%, such as at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1. According to other more particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 85%, such as at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1. According to other more particular

embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1. According to other more particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1.

According to particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 2. According to more particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 80%, such as at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 2. According to other more particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 85%, such as at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 2. According to other more particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 2. According to other more particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 2.

According to particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 3. According to more particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 80%, such as at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 3. According to other more particular embodiments, a

polypeptide according to ii) comprises an amino acid sequence which has at least about 85%, such as at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 3. According to other more particular embodiments, a polypeptide according to ii) comprises an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 3. According to other more particular embodiments, a polypeptide according to the invention comprises an amino acid sequence which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 3.

Preferably, a polypeptide according to ii) has tyrosine ammonia lyase activity. More preferably, a polypeptide according to ii) has a tyrosine ammonia lyase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1).

According to certain embodiment, a polypeptide according to ii) has tyrosine ammonia lyase activity similar to that of the polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 1. According to certain other embodiments, a polypeptide according to ii) has tyrosine ammonia lyase activity similar to that of the polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2. According to certain other embodiments, a polypeptide according to ii) has tyrosine ammonia lyase activity similar to that of the polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 3. With "similar" tyrosine ammonia lyase activity it is meant that the polypeptide according to ii) has at least about 10%, such as at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60, at least about 75%, at least about 80%, at least about 90%, at least about 95%, at least about 100%, at least about 200%, at least about 200%, at least about 400% or at least about 800%, of the ammonia lyase activity of the reference polypeptide (e.g., SEQ ID NO: 1).

The tyrosine ammonia lyase activity may for instance be determined in accordance to the following method: Enzymatic assays are performed in 200 µL volumes in wells in a UV transparent 96-well plate, by following the increase in absorbance at 315 nm (pHCA) using spectrophotometry or HPLC with UV detection. The reaction mixtures contain 2 µg of purified protein and are initiated by adding 1 mM tyrosine or 6 mM after equilibration to 30°C. The enzymatic activity is calculated as U/g, where U is defined as µmol substrate converted per minute. Negative controls contain no purified protein. Kinetic constants Km and vmax are

determined from assays containing 1.56 µM to 200 µM tyrosine. See also Kyndt et al. (2002).

As determined in accordance with Example 2, the values for K_m (µM), k_{cat} (min^{-1}) and k_{cat}/K_m (mM^{-1} s^{-1}) for the tyrosine ammonia lyase derived from Flavobacterium johnsoniae (SEQ ID NO: 1) using tyrosine as substrate are 5.7, 1.27 and 3.71, respectively. Each of these kinetic parameters may serve as reference parameter to determine the tyrosine ammonia lyase activity of the polypeptide according to ii), however, k_{cat}/K_m is preferred.

As determined in accordance with Example 2, the values for K_m (µM), k_{cat} (min^{-1}) and k_{cat}/K_m (mM^{-1} s^{-1}) for the tyrosine ammonia lyase derived from Herpetosiphon aurantiacus (SEQ ID NO: 2) using tyrosine as substrate are 16, 3.10 and 3.29, respectively. Each of these kinetic parameters may serve as reference parameter to determine the tyrosine ammonia lyase activity of the polypeptide according to ii), however, k_{cat}/K_m is preferred.

According to certain embodiments, a polypeptide according to ii) shows tyrosine ammonia lyase activity expressed as k_{cat}/K_m of at least about 3.2 mM^{-1} s^{-1}, such as at least about 3.25 mM^{-1} s^{-1}, at least about 3.29 mM^{-1} s^{-1}, at least about 3.5 mM^{-1} s^{-1}, at least about 3.6 mM^{-1} s^{-1}, at least about 3.65 mM^{-1} s^{-1} or at least about 3.7 mM^{-1} s^{-1}.

According to certain embodiments, a polypeptide according to ii) has an affinity (Km) towards phenylalanine of at least about 4000 µM, such as at least about 5000 µM, at least about 6000 µM or at least about 6500 µM.

For improved substrate specificity towards tyrosine, a polypeptide according to ii) preferably comprises the amino acid sequence set forth in SEQ ID NO: 4 or 5. The sequence LIRSHSSG (SEQ ID NO: 4) defines the region within the tyrosine ammonia lyase derived from Flavobacterium johnsoniae (SEQ ID NO: 1) conferring the substrate specificity towards tyrosine, whereas the sequence AIWYHKTG (SEQ ID NO: 5) defines the region within the tyrosine ammonia lyases derived from Herpetosiphon aurantiacus (SEQ ID NO: 2 or 3) conferring the substrate specificity towards tyrosine. Therefore, according to certain embodiments, a polypeptide according to ii) comprises the amino acid sequence set forth in SEQ ID NO: 4. According to certain other embodiments, a polypeptide according to ii) comprises the amino acid sequence set forth in SEQ ID NO: 5.

According to other certain embodiments, a polypeptide according to the invention is a polypeptide according to iii). Accordingly, a polypeptide according to the invention may be a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1), wherein 1 or more, such as 2 or more, 3 or more, 4 or more, 5 or more, 6 or more,

7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 25 or more, 30 or more, 35 or more, 40 or more, 45 or more, 50 or more, 60 or more, 70 or more, 80 or more, 90 or more, 100 or more, 110 or more, 120 or more, 130 or more, 140 or more, or 150 or more, amino acid residues are substituted, deleted, and/or inserted. According to particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1), wherein about 1 to about 150, such as about 1 to about 140, about 1 to about 130, about 1 to about 120, about 1 to about 110, about 1 to about 100, about 1 to about 90, about 1 to about 80, about 1 to about 70, about 1 to about 60, about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1), wherein about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to other more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1), wherein about 1 to about 30, such as about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to other more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1), wherein about 1 to about 25, such as about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted.

According to particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 1, wherein about 1 to about 150, such as about 1 to about 140, about 1 to about 130, about 1 to about 120, about 1 to about 110, about 1 to about 100, about 1 to about 90, about 1 to about 80, about 1 to about 70, about 1 to about 60, about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 1, wherein about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20,

about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to other more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 1, wherein about 1 to about 30, such as about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to other more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 1, wherein about 1 to about 25, such as about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted.

According to other particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 2, wherein about 1 to about 150, such as about 1 to about 140, about 1 to about 130, about 1 to about 120, about 1 to about 110, about 1 to about 100, about 1 to about 90, about 1 to about 80, about 1 to about 70, about 1 to about 60, about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 2, wherein about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to other more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 2, wherein about 1 to about 30, such as about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to other more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 2, wherein about 1 to about 25, such as about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted.

According to particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 3, wherein about 1 to about 150, such as about 1 to about 140, about 1 to about 130, about 1 to about 120, about 1 to about 110, about 1 to about 100, about 1 to about 90, about 1 to about 80, about 1 to about 70, about 1 to about 60, about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to

about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 3, wherein about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to other more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 3, wherein about 1 to about 30, such as about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted. According to other more particular embodiments, a polypeptide according to iii) comprises an amino acid sequence set forth in SEQ ID NO: 3, wherein about 1 to about 25, such as about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted.

It is understood that the foregoing values generally define the total number of alterations to the reference polypeptide (i.e. SEQ ID NO: 1, 2 or 3). The alterations may solely be amino acid substitutions, be it conserved or non-conserved substitutions, or both. They may solely be amino acid deletions. They may solely be amino acid insertions. The alterations may be a mix of these specific alterations, such as amino acid substitutions and amino acid insertions.

Preferably, a polypeptide according to iii) has tyrosine ammonia lyase activity. More preferably, a polypeptide according to iii) has a tyrosine ammonia lyase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1). According to certain embodiment, a polypeptide according to iii) has tyrosine ammonia lyase activity similar to that of the polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 1. According to certain other embodiments, a polypeptide according to iii) has tyrosine ammonia lyase activity similar to that of the polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2. According to certain other embodiments, a polypeptide according to iii) has tyrosine ammonia lyase activity similar to that of the polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 3. With "**similar**" tyrosine ammonia lyase activity it is meant that the polypeptide according to iii) has at least about 10%, such as at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60, at least about 75%, at least about 80%, at least about 90%, at least about 95%, at least about 100%, at least about 200%, at least about 200%, at least about 400% or at least about 800%, of the ammonia lyase activity of the reference polypeptide (i.e. SEQ ID NO: 1, 2 or 3).

The tyrosine ammonia lyase activity may for instance be determined in accordance to the following method: Enzymatic assays are performed in 200 µL volumes in wells in a UV transparent 96-well plate, by following the increase in absorbance at 315 nm (pHCA) using spectrophotometry or HPLC with UV detection. The reaction mixtures contain 2 µg of purified protein and are initiated by adding 1 mM tyrosine or 6 mM after equilibration to 30°C. The enzymatic activity is calculated as U/g, where U is defined as µmol substrate converted per minute. Negative controls contain no purified protein. Kinetic constants Km and vmax are determined from assays containing 1.56 µM to 200 µM tyrosine. See also Kyndt et al. (2002).

As shown in Example 2, the values for K_m (µM), k_{cat} (min^{-1}) and k_{cat}/K_m (mM^{-1} s^{-1}) for the tyrosine ammonia lyase derived from Flavobacterium johnsoniae (SEQ ID NO: 1) using tyrosine as substrate are 5.7, 1.27 and 3.71, respectively. Each of these kinetic parameters may serve as reference parameter to determine the tyrosine ammonia lyase activity of the polypeptide according to iii), however, k_{cat}/K_m is preferred.

As shown in Example 2, the values for K_m (µM), k_{cat} (min^{-1}) and k_{cat}/K_m (mM^{-1} s^{-1}) for the tyrosine ammonia lyase derived from Herpetosiphon aurantiacus (SEQ ID NO: 2) using tyrosine as substrate are 16, 3.10 and 3.29, respectively. Each of these kinetic parameters may serve as reference parameter to determine the tyrosine ammonia lyase activity of the polypeptide according to iii), however, k_{cat}/K_m is preferred.

According to certain embodiments, a polypeptide according to iii) shows tyrosine ammonia lyase activity expressed as k_{cat}/K_m of at least about 3.2 mM^{-1} s^{-1}, such as at least about 3.25 mM^{-1} s^{-1}, at least about 3.29 mM^{-1} s^{-1}, at least about 3.5 mM^{-1} s^{-1}, at least about 3.6 mM^{-1} s^{-1}, at least about 3.65 mM^{-1} s^{-1} or at least about 3.7 mM^{-1} s^{-1}.

According to certain embodiments, a polypeptide according to iii) has an affinity (Km) towards phenylalanine of at least about 4000 µM, such as at least about 5000 µM, at least about 6000 µM or at least about 6500 µM.

For improved substrate specificity towards tyrosine, a polypeptide according to iii) preferably comprises the amino acid sequence set forth in SEQ ID NO: 4 or 5. The sequence LIRSHSSG (SEQ ID NO: 4) defines the region within the tyrosine ammonia lyase derived from Flavobacterium johnsoniae (SEQ ID NO: 1) conferring the substrate specificity towards tyrosine, whereas the sequence AIWYHKTG (SEQ ID NO: 5) defines the region within the tyrosine ammonia lyase derived from Herpetosiphon aurantiacus (SEQ ID NO: 2 or 3) conferring the substrate specificity towards tyrosine. Therefore, according to certain embodiments, a polypeptide according to iii) comprises the amino acid sequence set forth in

SEQ ID NO: 4. According to certain other embodiments, a polypeptide according to iii) comprises the amino acid sequence set forth in SEQ ID NO: 5.

The polypeptide may be employed in accordance with the invention in isolated form, such as in purified form. The polypeptide may for instance be expressed by a recombinant host cell, and then purified. Techniques and means for the purification of polypeptides produced by a recombinant host cell are well know in the art. For example, in order to facilitate purification, an amino acid motif comprising several histidine residues, such as at least 6, may be inserted at the C- or N-terminal end of the polypeptide. A non-limiting example of such amino acid motif is provided in SEQ ID NO: 11. Various purification kits for histidine-tagged polypeptides are available from commercial sources such as Qiagen, Hilden, Germany; Clontech, Mountain View, CA, USA; Bio-Rad, Hercules, CA, USA and others.

Alternatively, The polypeptide may be chemically synthezised. Techniques for chemical peptide synthesis are well know and include Liquid-phase synthesis and Solid-phase synthesis.

The polypeptide can also be employed in accordance with the invention as part of a recombinant host cell. Such recombinant host cells are described in more details below.

It is understood that the details given herein with respect to a polypeptide apply to all aspects of the invention.

The present invention also provides a recombinant host cell comprising (e.g. expressing) a polypeptide as detailed herein. Generally, the polypeptide according to the invention will be heterologous to the host cell, which means that the polypeptide is normally not found in or made (i.e. expressed) by the host cell, but derived from a different species.

Therefore, the present invention provides a recombinant host cell according to the present invention comprises a heterologous polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1);

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1); or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1), wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 1 to 3, amino acid residues are substituted, deleted and/or inserted.

Recombinant host cells in accordance with the invention can be produced from any suitable host organism, including single-celled or multicellular microorganisms such as bacteria, yeast, fungi, algae and plant, and higher eukaryotic organisms including nematodes, insects, reptiles, birds, amphibians and mammals.

Bacterial host cells are selected from Gram-positive and Gram-negative bacteria. Non-limiting examples for Gram-negative bacterial host cells include species from the genera *Escherichia, Erwinia, Klebsiella* and *Citrobacter*. Non-limiting examples of Gram-positive bacterial host cells include species from the genera *Bacillus, Lactococcus, Lactobacillus, Clostridium, Corynebacterium, Streptomyces, Streptococcus*, and *Cellulomonas*.

According to certain embodiments, the recombinant host cell is a bacterium, which may be a bacterium of the genus *Bacillus, Lactococcus, Lactobacillus, Clostridium, Corynebacterium, Geobacillus, Streptococcus, Pseudomonas, Streptomyces, Escherichia, Shigella, Acinetobacter, Citrobacter, Salmonella, Klebsiella, Enterobacter, Erwinia, Kluyvera, Serratia, Cedecea, Morganella, Hafnia, Edwardsiella, Providencia, Proteus,* or *Yersinia*.

According to particular embodiments, the recombinant host cell is a bacterium of the genus *Bacillus*. Non-limiting examples of a bacteria of the genus *Bacillus* are *Bacillus subtitlis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus mojavensis*. According to more particular embodiments, the recombinant host cell is *Bacillus subtitlis*. According to other more particular embodiments, the recombinant host cell is *Bacillus licheniformis*.

According to other particular embodiments, the recombinant host cell is a bacterium of the genus *Lactococcus*. A non-limiting example of a bacterium of the genus *Lactococcus* is *Lactococcus lactis*. According to more particular embodiments, the recombinant host cell is *Lactococcus lactis*.

According to other particular embodiments, the recombinant host cell is a bacterium of the genus *Corynebacterium*. A non-limiting example of a bacterium of the genus *Corynebacterium* is *Corynebacterium glutamicum*. According to more particular embodiments, the recombinant host cell is *Corynebacterium glutamicum*.

According to other particular embodiments, the recombinant host cell is a bacterium of the genus *Streptomyces*. A non-limiting examples of a bacterium of the genus *Streptomyces* are

Streptomyces lividans, Streptomyces coelicolor, or *Streptomyces griseus*. According to more particular embodiments, the recombinant host cell is *Streptomyces lividans*. According to other more particular embodiments,, the recombinant host cell is *Streptomyces coelicolor*. According to other more particular embodiments,, the recombinant host cell is *Streptomyces griseus.*

According to other particular embodiments, the recombinant host cell is a bacterium of the genus *Pseudomonas*. A non-limiting example of a bacterium of the genus *Pseudomonas* is *Pseudomonas putida*. According to more particular embodiments, the recombinant host cell is *Pseudomonas putida*.

According to other particular embodiments, the recombinant host cell is a bacterium of the genus *Escherichia*. A non-limiting example of a bacterium of the genus *Escherichia* is *Escherichia coli*. According to more particular embodiments, the recombinant host cell is *Escherichia coli*.

Yeast host cells may be derived from e.g., *Saccharomyces, Pichia, Schizosacharomyces, Zygosaccharomyces, Hansenula, Pachyosolen, Kluyveromyces, Debaryomyces, Yarrowia, Candida, Cryptococcus, Komagataella, Lipomyces, Rhodospiridium, Rhodotorula*, or *Trichosporon*.

According to certain embodiments, the recombinant host cell is a yeast, which may be a yeast is of the genus *Saccharomyces, Pichia, Schizosacharomyces, Zygosaccharomyces, Hansenula, Pachyosolen, Kluyveromyces, Debaryomyces, Yarrowia, Candida, Cryptococcus, Komagataella, Lipomyces, Rhodospiridium, Rhodotorula*, or *Trichosporon*.

According to particular embodiments, the recombinant host cell is a yeast of the genus *Saccharomyces*. A non-limiting example of a yeast of the genus *Saccharomyces* is *Saccharomyces cerevisiae*. According to more particular embodiments, the recombinant host cell is *Saccharomyces cerevisiae*.

According to particular embodiments, the recombinant host cell is a yeast of the genus *Pichia*. Non-limiting example of a yeast of the genus *Pichia* are *Pichia pastoris* and *pichia kudriavzevii*. According to more particular embodiments, the recombinant host cell is *Pichia pastoris*. According to other more particular embodiments, the recombinant host cell is *pichia kudriavzevii*.

Fungi host cells may be derived from, e.g., *Aspergillus.*

According to certain embodiments, the recombinant host cell is a fungus, such as a fungi of the genus *Aspergillus*. Non-limiting examples of a fungus of the genus *Aspergillus* are *Aspergillus Oryzae, Aspergillus niger* or *Aspergillus awamsii.* According to more particular embodiments, the recombinant host cell is *Aspergillus Oryzae*. According to other more particular embodiments, the recombinant host cell is *Aspergillus niger*. According to other more particular embodiments, the recombinant host cell is *Aspergillus awamsii*.

Algae host cells may be derived from, e.g., *Chlamydomonas, Haematococcus, Phaedactylum, Volvox* or *Dunaliella*.

According to certain embodiments, the recombinant host cell is an alga, which may be an algae of the genus *Chlamydomonas, Haematococcus, Phaedactylum, Volvox* or D*unaliella*.

According to particular embodiments, the recombinant host cell is an alga cell of the genus *Chlamydomonas*. A non-limiting example of an alga of the genus *Chlamydomonas* is *Chlamydomonas reinhardtii*.

According to particular embodiments, the recombinant host cell is an alga cell of the genus *Haematococcus*. A non-limiting example of an alga of the genus *Haematococcus* is *Haematococcus pluvialis*.

According to other particular embodiments, the recombinant host cell is an alga cell of the genus *Phaedactylum*. A non-limiting example of an alga of the genus *Phaedactylum* is *Phaedactylum tricornatum*.

A plant host cell may be derived from, e.g., soybean, rapeseed, sunflower, cotton, corn, tobacco, alfalfa, wheat, barley, oats, sorghum, lettuce, rice, broccoli, cauliflower, cabbage, parsnips, melons, carrots, celery, parsley, tomatoes, potatoes, strawberries, peanuts, grapes, grass seed crops, sugar beets, sugar cane, beans, peas, rye, flax, hardwood trees, softwood trees, and forage grasses.

According to certain embodiments, the recombinant host cell is a plant cell, such as a plant cell selected from the group consisting of soybean, rapeseed, sunflower, cotton, corn, tobacco, alfalfa, wheat, barley, oats, sorghum, lettuce, rice, broccoli, cauliflower, cabbage, parsnips, melons, carrots, celery, parsley, tomatoes, potatoes, strawberries, peanuts, grapes, grass seed crops, sugar beets, sugar cane, beans, peas, rye, flax, hardwood trees, softwood trees, and forage grasses.

Generally, a recombinant host cell according to the invention has been genetically modified to express a polypeptide as detailed herein, which means that an exogenous nucleic acid

molecule, such as a DNA molecule, which comprises a nucleotide sequence encoding said polypeptide has been introduced in the host cell. Techniques for introducing exogenous nucleic acid molecule, such as a DNA molecule, into the various host cells are well-known to those of skill in the art, and include transformation (e.g., heat shock or natural transformation), transfection, conjugation, electroporation and microinjection.

Accordingly, a host cell according to the invention comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide as detailed herein.

In order to facilitate expression of the polypeptide in the host cell, the exogenous nucleic acid molecule may comprise suitable regulatory elements such as a promoter that is functional in the host cell to cause the production of an mRNA molecule and that is operably linked to the nucleotide sequence encoding said polypeptide.

Promoters useful in accordance with the invention are any known promoters that are functional in a given host cell to cause the production of an mRNA molecule. Many such promoters are known to the skilled person. Such promoters include promoters normally associated with other genes, and/or promoters isolated from any bacteria, yeast, fungi, alga or plant cell. The use of promoters for protein expression is generally known to those of skilled in the art of moleculer biology, for example, see Sambrook et al., Molecular cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. , 1989. The promoter employed may be inducible. The term "inducible" used in the context of a promoter means that the promoter only directs transcription of an operably linked nucleotide sequence if a stimulus is present, such as a change in temperature or the presence of a chemical substance ("chemical inducer"). As used herein, "chemical induction" according to the present invention refers to the physical application of a exogenous or endogenous substance (incl. macromolecules, e.g., proteins or nucleic acids) to a host cell. This has the effect of causing the target promoter present in the host cell to increase the rate of transcription. Alternatively, the promoter employed may be constitutive. The term "constitutive" used in the context of a promoter means that the promoter is capable of directing transcription of an operably linked nucleotide sequence in the absence of stimulus (such as heat shock, chemicals etc.).

Non-limiting examples of promoters functional in bacteria, such as *Bacillus subtilis, Lactococcus lactis* or *Escherichia coli*, include both constitutive and inducible promoters such as T7 promoter, the beta-lactamase and lactose promoter systems; alkaline phosphatase (phoA) promoter, a tryptophan (trp) promoter system, tetracycline promoter,

lambda-phage promoter, ribosomal protein promoters; and hybrid promoters such as the tac promoter. Other bacterial and synthetic promoters are also suitable.

Non-limiting examples of promoters functional in yeast, such as *Saccharomyces cerevisiae*, include xylose promoter, GAL1 and GAL10 promoters, TEF1 promoter, and pgk1 promoter.

Non-limiting examples of promoters functional in fungi, such as *Aspergillus Oryzae* or *Aspergillus niger*, include promotors derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral α-amylase, *Aspergillus niger* acid stable α-amylase, *Aspergillus niger* or *Aspergillus awamsii* glucoamylase (gluA), *Aspergillus niger* acetamidase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphatase isomerase, *Rhizopus meihei* aspartic proteinase, and *Rhizopus meihei* lipase.

Non-limiting examples of promoters functional in alga, such as *Haematococcus pluvialis*, include the CaMV35S promoter, the SV40 promoter, and promoter of the *Chlamydomonas reinhardtii* RBCS2 gene and the promoter of the *Volvox carteri* ARS gene.

Non-limiting examples of promoters functional in plant cells include the *Lactuca sative* psbA promoter, the tabacco psbA promoter, the tobacco rrn16 PEP+NEP promoter, the CaMV 35S promoter, the 19S promoter, the tomate E8 promoter, the nos promoter, the Mac promoter, and the pet E promoter or the ACT1 promoter.

Besides a promoter, the exogenous nucleic acid molecule may further comprise at least one regulatory element selected from a 5' untranslated region (5'UTR) and 3' untranslated region (3' UTR). Many such 5' UTRs and 3' UTRs derived from prokaryotes and eukaryotes are well known to the skilled person. Such regulatory elements include 5' UTRs and 3' UTRs normally associated with other genes, and/or 5' UTRs and 3' UTRs isolated from any bacteria, yeast, fungi, alga or plant cell.

If the host cell is a prokaryotic organism, the 5' UTR usually contains a ribosome binding site (RBS), also known as the Shine Dalgarno sequence which is usually 3-10 base pairs upstream from the initiation codon. Meanwhile, if the host cell is an eukaryotic organism the 5' UTR usually contains the Kozak consensus sequence. An eukaryotic 5' UTR may also contain cis-acting regulatory elements.

The exogenous nucleic acid molecule may be a vector or part of a vector, such as an expression vector. Normally, such a vector remains extrachromosomal within the host cell which means that it is found outside of the nucleus or nucleoid region of the host cell.

It is also contemplated by the present invention that the exogenous nucleic acid molecule is stably integrated into the genome of the host cell. Means for stable integration into the genome of a host cell, e.g., by homologous recombination, are well known to the skilled person.

In order to prevent degradation of the hydroxycinnamic acids produced by a method of the present invention involving the use of recombinant host cells, a recombinant host cell, especially a recombinant bacterial host cell such as *Bacillus subtilis* or *Lactococcus lactis*, may further be genetically modified by inactivating a gene or gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity. **By "inactivating" or "inactivation of" a gene** or gene cluster it is intended that the gene or cluster of interest (e.g. the gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity) is not expressed in a functional protein form. Techniques for inactivating a gene or gene cluster are well-known to those of skill in the art, and include random mutagenesis, site specific mutagenesis, recombination, integration and others.

According to certain embodiments, the recombinant host cell does not express a polypeptide having phenolic acid decarboxylase (PAD) activity.

According to cartain embodiments, the recombinant host cell has been genetically modified to inactivate a gene or gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity.

According to particular embodiments, the recombinant host cell is a bacterium of the genus *bacillus,* such as *bacillus subtiltis,* or *lactococcus,* such as *lactococcus lactis,* which has been genetically modified to inactivate the padC (or padA) gene.

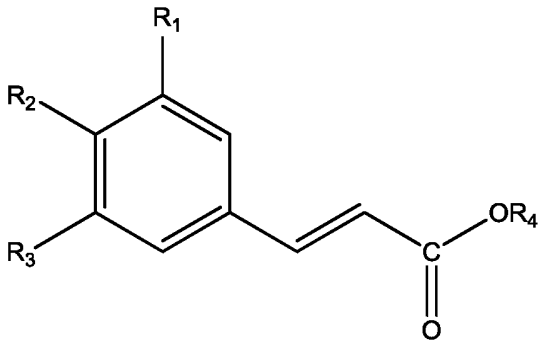
According to other particular embodiments, the recombinant host cell is a yeast of the genus *Saccharomyces, such as Saccharomyces cerevisiae*, which has been genetically modified to inactivate the pad1 gene.

According to other certain embodiments, the recombinant host cell does not contain within its genome a gene or gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity.

It is understood that the details given herein with respect to a recombinant host cell apply to other aspects of the invention, in particular to the methods and uses according to the invention, which are described in more detail below.

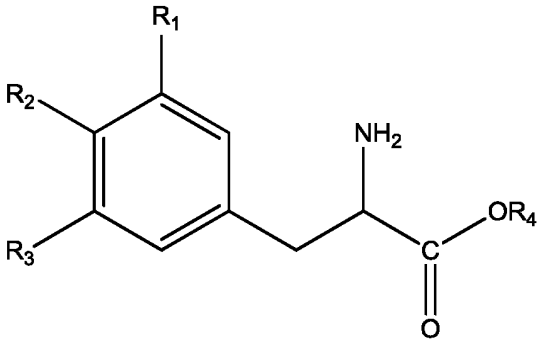
Methods and uses

The present invention provides methods and uses for producing hydroxycinnamic acids. Particularly, a method for producing a hydroxycinnamic acid of general formula I



general formula I

, the method comprises deaminating a compound of general formula II



general formula II

using a polypeptide as detailed herein, which may be selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g., SEQ ID NO: 1);

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g. SEQ ID NO: 1); or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3 (e.g. SEQ ID NO: 1), wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to

about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted;

wherein R_1, R_2 and R_3 independently are selected from the group consisting of hydrogen (H), hydroxyl (-OH), C_{1-6}-alkyl and C_{1-6}-Alkoxy, provided that at least one of R_1, R_2 and R_3 is hydroxyl (-OH); and R_4 is selected from the group consisting of hydrogen (-H) and C_{1-6}-alkyl.

"Deamination" or "deaminating" as used herein means that the amine group on the alpha carbon atom in the compound according to general formula II is removed.

Within the context of the present invention, R_1 may be hydrogen, hydroxyl, C_{1-6}-alkyl or C_{1-6}-Alkoxy. According to certain embodiments, R_1 is hydrogen. According to other certain embodiments, R_1 is hydroxyl. According to other certain embodiments, R_1 is C_{1-6}-alkyl, such as methyl or ethyl. According to other certain embodiments, R_1 is C_{1-6}-Alkoxy, such as methoxyl ($-OCH_3$).

Within the context of the present invention, R_2 may be hydrogen, hydroxyl, C_{1-6}-alkyl or C_{1-6}-Alkoxy. According to certain embodiments, R_2 is hydrogen. According to other certain embodiments, R_2 is hydroxyl. According to other certain embodiments, R_2 is C_{1-6}-alkyl, such as methyl or ethyl. According to other certain embodiments, R_2 is C_{1-6}-Alkoxy, such as methoxyl ($-OCH_3$).

Within the context of the present invention, R_3 may be hydrogen, hydroxyl, C_{1-6}-alkyl or C_{1-6}-Alkoxy. According to certain embodiments, R_3 is hydrogen. According to other certain embodiments, R_3 is hydroxyl. According to other certain embodiments, R_3 is C_{1-6}-alkyl, such as methyl or ethyl. According to other certain embodiments, R_3 is C_{1-6}-Alkoxy, such as methoxyl ($-OCH_3$).

Within the context of the present invention, R_4 may be hydrogen or C_{1-6}-alkyl. According to certain embodiments, R_4 is hydrogen. According to other certain embodiments, R_4 is C_{1-6}-alkyl, such as methyl ($-CH_3$) or ethyl ($-CH_2CH_3$).

According to particular embodiments, the method is for producing p-coumaric acid (R_1=H, R_2=OH, R_3=H, R_4=H), caffeic acid (R_1=H, R_2=OH, R_3=OH, R_4=H), ferulic acid (R_1=OCH_3, R_2=OH, R_3=H, R_4=H) or sinapic acid (R_1= OCH_3, R_2=OH, R3= OCH_3, R4=H). According to more particular embodiments, the method is for producing p-coumaric acid (R_1=H, R_2=OH, R_3=H, R4=H). According to other more particular embodiments, the method is for producing of caffeic acid (R_1=H, R_2=OH, R_3=OH, R_4=H). According to other more particular embodiments, the method is for producing ferulic acid (R_1=OCH_3, R_2=OH, R_3=H, R_4=H).

According to other more particular embodiments, the method is for producing sinapic acid (R_1= OCH_3, R_2=OH, R_3= OCH_3, R_4=H).

Suitable conditions for the deamination reaction are well known to the skilled person. Typically, the deamination reaction takes place at a temperature ranging from about 23 to about 60°C, such as from about 25 to about 40°C, such as at about 37°C. The deamination reaction may take place at a pH ranging from pH 4.0 to pH 14.0, such as from about pH 6 to about pH 11, or from about pH 7 to about pH 9.5, e.g. at pH 6.0, pH pH 7.0, pH. 7.5, pH 8.0, pH 8.5, pH 9.0, pH 9.5, pH 10.0, pH 10.5 or pH 11.0.

Moreover, the present invention provides a method for producing a hydroxcinnamic acid of general formula I as defined above, the method comprises the step of:

a) contacting a recombinant host cell as detailed herein with a medium comprising a fermentable carbon substrate and/or a compound of the general formula II as defined above.

The medium employed may be any conventional medium suitable for culturing the host cell in question, and may be composed according to the principles of the prior art. The medium will usually contain all nutrients necessary for the growth and survival of the respective host cell, such as carbon and nitrogen sources and other inorganic salts. Suitable media, e.g. minimal or complex media, are available from commercial suppliers, or may be prepared according to published receipts, e.g. the American Type Culture Collection (ATCC) Catalogue of strains. Non-limiting standard medium well known to the skilled person include Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth, MS broth, Yeast Peptone Dextrose, BMMY, GMMY, or Yeast Malt Extract (YM) broth, which are all commercially available. A non-limiting example of suitable media for culturing bacterial cells, such as B. subtilis, L. lactis or E. coli cells, including minimal media and rich media such as Luria Broth (LB), M9 media, M17 media, SA media, MOPS media, Terrific Broth, YT and others. Suitable media for culturing eukaryotic cells, such as yeast cells, are RPMI 1640, MEM, DMEM, all of which may be supplemented with serum and/or growth factors as required by the particular host cell being cultured. The medium for culturing eukaryotic cells may also be any kind of minimal media such as Yeast minimal media.

The fermentable carbon substrate may be any suitable carbon substrate know in the art, and in particularly any carbon substrate commonly used in the cultivation of microorganisms and/ or fermentation. Non-limiting examples of suitable fermentable carbon substates include carbohydrates (e.g., C5 sugars such as arabinose or xylose, or C6 sugars such as glucose), glycerol, glycerine, acetate, dihydroxyacetone, one-carbon source, methanol, methane, oils,

animal fats, animal oils, plant oils, fatty acids, lipids, phospholipids, glycerolipids, monoglycerides, diglycerides, triglycerides, renewable carbon sources, polypeptides (e.g., a microbial or plant protein or peptide), yeast extract, component from a yeast extract, peptone, casaminoacids or any combination of two or more of the foregoing.

According to certain embodiments, the carbon substate is selected from the group consisting of C5 sugars (such as arabinose or xylose), C6 sugars (such as glucose or fructose), lactose, sucrose, glycerol, glycerine, acetate, yeast extract, component from a yeast extract, peptone, casaminoacids or combinations thereof.

According to certain embodiments, the medium comprises glucose.

According to certain other embodiments, the medium comprises glycerol.

According to certain other embodiments, the medium comprises acetate.

It is also contemplated to use starch as a carbon substrate. Depending on the microorganism used, the metabolization of starch may require the supplementation of beta-glucosidase, such as the beta-glucosidase from Neurospora crassa, to the medium. Alternatively, a recombination host cell according to the invention may be further genetically modified to express a beta-glucosidase, such as the beta-glucosidase from Neurospora crassa.

When a fermentable carbon substrate is employed it is thus possible that the recombinant host cell produces the hydroxycinnamic acid according to the invention directly from such primary carbon substrate.

Therefore, according to certain embodiments, the method for producing a hydroxcinnamic acid of general formula I as defined above comprises the step of:

a) contacting a recombinant host cell as detailed herein with a medium comprising a fermentable carbon substrate.

According to certain other embodiments, the method for producing a hydroxcinnamic acid of general formula I as defined above comprises the step of:

a) contacting a recombinant host cell as detailed herein with a medium comprising a compound of the general formula II as defined above.

According to certain other embodiments, the method for producing a hydroxcinnamic acid of general formula I as defined above comprises the step of:

a) contacting a recombinant host cell as detailed herein with a medium comprising a fermentable carbon substrate and a compound of the general formula II as defined above.

The addition of exogenous tyrosine to the medium has shown to increase the production yield of the hydroxcinnamic acid (notably p-coumaric acid). See Table 4 below.

The method may further comprise step b) culturing the recombinant host cell under suitable conditions for the production of the hydroxcinnamic acid.

Suitable conditions for culturing the respective host cell are well known to the skilled person. Typically, the recombinant host cell is cultured at a temperature ranging from about 23 to about 60°C, such as from about 25 to about 40°C, such as at about 37°C. The pH of the medium may range from pH 4.0 to pH 14.0, such as from about pH 6 to about pH 11, or from about pH 7 to about pH 9.5, e.g. at pH 6.0, pH pH 7.0, pH. 7.5, pH 8.0, pH 8.5, pH 9.0, pH 9.5, pH 10.0, pH 10.5 or pH 11.0.

The method may further comprise step c) recovering the hydroxcinnamic acid. The hydroxcinnamic acid may be recovered by conventional method for isolation and purification chemical compounds from a medium. Well-known purification procedures include centrifugation or filtration, precipitation, and chromatographic methods such as e.g. ion exchange chromatography, gel filtration chromatography, etc.

The present invention further provides the use of a polypeptide as detailed herein in the production of a hydroxycinnamic acid, and particularly in the production of a hydroxycinnamic acid is of the general formula I. According to more particular embodiments, the present invention provides the use a polypeptide as detailed herein in the production of p-coumaric acid.

Certain definitions

"**Tyrosine ammonia lyase activity**" as used herein refers to the ability of a polypeptide to catalysed the conversion of L-tyrosine into p-coumaric acid.

"Polypeptide," and "protein" are used interchangeably herein to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-transiational modification (e.g., glycosylation, phosphorylation, lipidation, myristilation, ubiquitination, etc.). Included within this definition are D- and L-amino acids, and mixtures of D- and L-amino acids.

"Nucleic acid" or "polynucleotide" are used interchangeably herein to denote a polymer of at least two nucleic acid monomer units or bases (e.g., adenine, cytosine, guanine, thymine) covalently linked by a phosphodiester bond, regardless of length or base modification.

"Recombinant" or "non-naturally occurring" when used with reference to, e.g., a host cell, nucleic acid, or polypeptide, refers to a material, or a material corresponding to the natural or native form of the material, that has been modified in a manner that would not otherwise exist in nature, or is identical thereto but produced or derived from synthetic materials and/or by manipulation using recombinant techniques. Non-limiting examples include, among others, recombinant host cells expressing genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise expressed at a different level.

"Substitution" or "substituted" refers to modification of the polypeptide by replacing one amino acid residue with another, for instance the replacement of an Arginine residue with a Glutamine residue in a polypeptide sequence is an amino acid substitution.

"Conservative substitution" refers to a substitution of an amino acid residue with a different residue having a similar side chain, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar class of amino acids. By way of example and not limitation, an amino acid with an aliphatic side chain may be substituted with another aliphatic amino acid, e.g., alanine, valine, leucine, and isoleucine; an amino acid with hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain, e.g., serine and threonine; an amino acid having an aromatic side chain is substituted with another amino acid having an aromatic side chain, e.g., phenylalanine, tyrosine, tryptophan, and histidine; an amino acid with a basic side chain is substituted with another amino acid with a basic side chain, e.g., lysine and arginine; an amino acid with an acidic side chain is substituted with another amino acid with an acidic side chain, e.g., aspartic acid or glutamic acid; and a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively.

"Non-conservative substitution" refers to substitution of an amino acid in a polypeptide with an amino acid with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and affects (a) the structure of the peptide backbone in the area of the substitution (e.g., proline for glycine) (b) the charge or hydrophobicity, or (c) the bulk of the side chain. By way of example and not limitation, an exemplary non-conservative substitution can be an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid

substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

"Deletion" or "deleted" refers to modification of the polypeptide by removal of one or more amino acids in the reference polypeptide. Deletions can comprise removal of 1 or more amino acids, 2 or more amino acids, 5 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, or up to 20% of the total number of amino acids making up the polypeptide while retaining enzymatic activity and/or retaining the improved properties of an engineered enzyme. Deletions can be directed to the internal portions and/or terminal portions of the polypeptide, in various embodiments, the deletion can comprise a continuous segment or can be discontinuous.

"Insertion" or "inserted" refers to modification of the polypeptide by addition of one or more amino acids to the reference polypeptide. Insertions can comprise addition of 1 or more amino acids, 2 or more amino acids, 5 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids. Insertions can be in the internal portions of the polypeptide, or to the carboxy or amino terminus. The insertion can be a contiguous segment of amino acids or separated by one or more of the amino acids in the reference polypeptide.

"Host cell" as used herein refers to a living cell or microorganism that is capable of reproducing its genetic material and along with it recombinant genetic material that has been introduced into it - e.g., via heterologous transformation.

"Expression" includes any step involved in the production of a polypeptide (e.g., encoded enzyme) including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

As used herein, "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid molecule to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded nucleic acid loop into which additional nucleic acid segments can be ligated. Certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". Certain other vectors are capable of facilitating the insertion of a exogenous nucleic acid molecule into a genome of a host cell. Such vectors are referred to herein as "transformation vectors". In general, vectors of utility in recombinant nucleic acid techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be

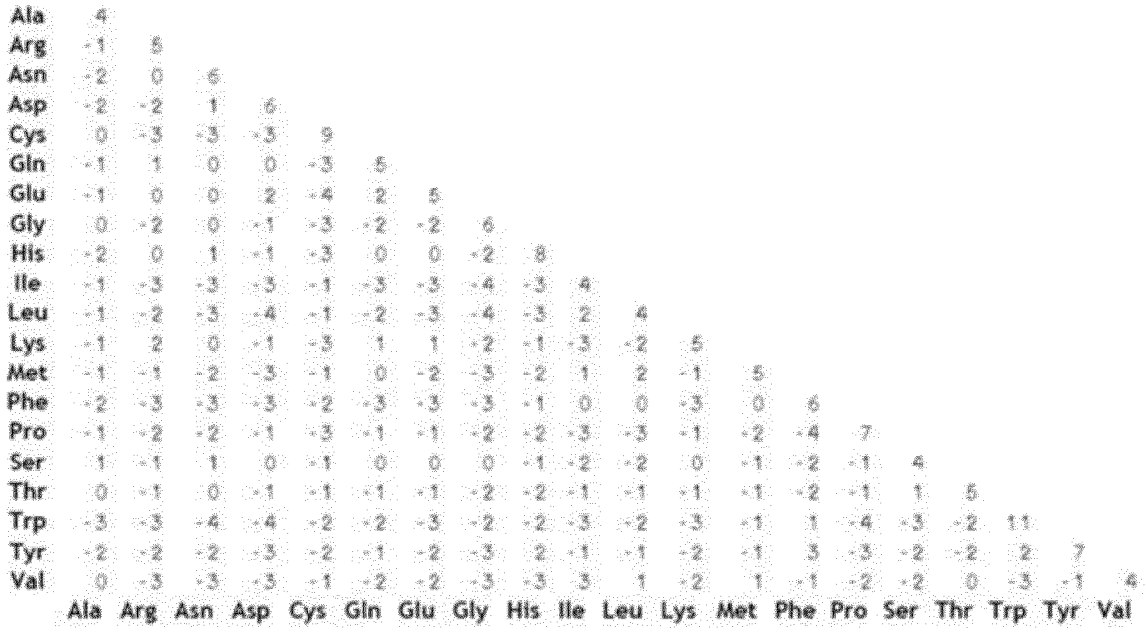
used interchangeably as the plasmid is the most commonly used form of a vector. Large numbers of suitable vectors are known to those of skill in the art and commercially available.

As used herein, "promoter" refers to a sequence of DNA, usually upstream (5') of the coding region of a structural gene, which controls the expression of the coding region by providing recognition and binding sites for RNA polymerase and other factors which may be required for initiation of transcription. The selection of the promoter will depend upon the nucleic acid sequence of interest. A "promoter functional in a host cell" refers to a "promoter" which is capable of supporting the initiation of transcription in said cell, causing the production of an mRNA molecule.

As used herein, "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequence. A promoter sequence is "operably-linked" to a gene when it is in sufficient proximity to the transcription start site of a gene to regulate transcription of the gene.

"Percentage of sequence identity," "% sequence identity" and "percent identity" are used herein to refer to comparisons between an amino acid sequence and a reference amino acid sequence. The "**% sequence identify**", as used herein, is calculated from the two amino acid sequences as follows: The sequences are aligned using Version 9 of the Genetic Computing Group's GAP (global alignment program), using the default BLOSUM62 matrix (see below) with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (for each additional null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the reference amino acid sequence.

The following BLOSUM62 matrix is used:



"Reference sequence" **or "reference amino acid sequence"** refers to a defined sequence to which another sequence is compared. In the context of the present invention a reference amino acid sequence may be an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3.

"Alkyl", "alkyl radical" or group as used herein means saturated, linear or branched hydrocarbons, which can be unsubstituted or mono- or polysubstituted. Thus, unsaturated alkyl is understood to encompass alkenyl and alkinyl groups, like e.g. -CH=CH-CH3 or -C=C-CH3, while saturated alkyl encompasses e.g. -CH3 and -CH2-CH3. **"C_{1-6}-alkyl" includes** C_{1-2}-alkyl, C_{1-3}-alkyl, C_{1-4}-alkyl, and C_{1-5}-alkyl, as well as C_{2-3}-alkyl, C_{2-4}-alkyl, C_{2-5}-alkyl, C_{3-4}-alkyl, C_{3-5}-alkyl, and C_{4-5}-alkyl. In these radicals, C_{1-2}-alkyl represents C_1- or C_2-alkyl, C_{1-3}-alkyl represents C_1-, C_2- or C_3-alkyl, C_{1-4}-alkyl represents C_1-, C_2-, C_3- or C_4-alkyl, C_{1-5}-alkyl represents C_1-, C_2-, C_3-, C_4-, or C_5-alkyl, C_{1-6}-alkyl represents C_1-, C_2-, C_3-, C_4-, C_5- or C_6-alkyl. The alkyl radicals may be methyl, ethyl, vinyl (ethenyl), propyl, allyl (2-propenyl), 1-propinyl, methylethyl, butyl, 1-methylpropyl, 2-methylpropyl, 1,1-dimethylethyl, pentyl, 1,1-dimethylpropyl, 1,2-dimethylpropyl, 2,2-dimethylpropyl, hexyl, 1-methylpentyl, if substituted also CHF2, CF3 or CH2OH etc.

"Alkoxy", "alkoxy radical" or group as used herein means an "alkyl" singular bonded to oxygen. **"C_{1-6}-alkoxy" includes** C_{1-2}- alkoxy, C_{1-3}-alkoxy, C_{1-4}-alkoxy, and C_{1-5}-alkoxy, as well as C_{2-3}-alkoxy, C_{2-4}-alkoxy, C_{2-5}-alkoxy, C_{3-4}-alkoxy, C_{3-5}-alkoxy, and C_{4-5}-alkoxy. In these radicals, C_{1-2}- alkoxy represents C1- or C2- alkoxy, C_{1-3}- alkoxy represents C_1-, C_2- or C_3-alkoxy, C_{1-4}-alkyl represents C_1-, C_2-, C_3- or C_4-alkoxy, C_{1-5}-alkoxy represents C_1-, C_2-, C_3-, C_4-, or C_5- alkoxy, C_{1-6}- alkoxy represents C_1-, C_2-, C_3-, C_4-, C_5- or C_6-alkoxy. The alkoxy radicals may be methoxy, ethoxy, propoxy, butoxy, pentyloxy or hexyloxy.

Where a numerical limit or range is stated herein, the endpoints are included. Also, all values and sub ranges within a numerical limit or range are specifically included as if explicitly written out.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples, which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

Examples

As demonstared in the following examples, the polypeptides according to the invention show higher TAL activity compared to previously characterized enzymes, in particularly when expressed in a commonly used production yeast, as well as in selected industrially relevant bacteria. They are active in Gram-positive bacteria, Gram-negative bacteria as wells as in eukaryotic microorganisms. The improved activities have also been shown in in vitro biochemical assays.

As further demonstrated below, the polypeptides according to the invention have very specific TAL activity over PAL activity. The polypeptides according to the invention thus allow the enhanced biologically production of hydroxycinnamic acids such as pHCA. Furthermore, the production can be enhanced by the disruption of degradation pathways and the addition of tyrosine either extracellularly.

Example 1 – Expression of TAL and PAL enzymes in E. coli

A number of previously described and newly identified enzymes were expressed in the Gram negative bacterium *E. coli* for the comparison of enzymatic activities.

A number of genes encoding aromatic amino acid lyases were codon optimized using standard algorithms for expression in *E. coli* available by GeneArt (Life Technologies). The enzymes are listed in table 1. RsTAL, RmXAL, SeSam8, TcXAL, PcXAL, and RtXAL have previously been described. FjXAL, HaXAL1 and HaXAL2 have not been described before.

Table 1. Overview of enzymes

Name	Organism	Protein GI	Len (aa)	SEQ ID NO
FjXAL	*Flavobacterium johnsoniae*	146298870	506	1
HaXAL1	*Herpetosiphon aurantiacus*	159898407	552	2
HaXAL2	*Herpetosiphon aurantiacus*	159898927	552	3
RsTAL	*Rhodobacter sphaeroides*	126464011	523	
RmXAL	*Rhodotorula mucilaginosa / Rhodotorula rubra*	129592	713	
SeSam8	*Saccharothrix espanaensis*	433607630	510	
RtXAL	*Rhodosporidium toruloides / Rhodotorula glutinis*	129593	716	
TcXAL	*Trichosporon cutaneum*	77375521	689	
PcXAL	*Phanerochaete chrysosporium*	259279291	737	

Each of the genes were amplified by polymerase chain reaction (PCR) using the primers indicated in Table 6. The final PCR products were inserted in the pCDFDuet-1 vector (Novagen / Life Technologies), which had been digested by NdeI and BglII using Gibson reaction (New England Biolabs) (selected plasmids are shown in Figures 1 to 3).

Plasmids carrying the genes were transformed into electrocompetent *E. coli* BL21(DE3)pLysS cells (Life Technologies) and selected on LB plates containing 50 ug/mL streptomycin. The strains were grown in M9 minimal media containing glucose as a carbon source, and expression was induced by adding 1 mM IPTG at an optical density at 600 nm of 0.6. After three hours of growth at 30°C the cultures were supplemented with 2 mM tyrosine, phenylalanine or histidine. After further 24 hours, samples were withdrawn for determination of the optical density at 600 nm and for the isolation of the supernatant.

The concentration of pHCA and CA in the supernatant was quantified by high performance (HPLC) and compared to chemical standards. HPLC was done on a Thermo setup using a HS-F5 column and mobile phases: 5 mM ammonium formate pH 4.0 (A) and acetonitrile (B) at 1.5 mL min-1, using a gradient elution starting at 5% B. From 0.5 min after injection to 7 min, the fraction of B increased linearly from 5% to 60%, and between 9.5 min and 9.6 the

fraction of B decreased back to 5%, and remaining there until 12 min. pHCA and CA were quantified by measuring absorbance at 333 nm and 277 nm, respectively. The production was tested without addition of precursors or the addition of either phenylalanine or tyrosine to the growth medium.

Table 2 shows the specific production of pHCA and CA in the various media. The specific production was calculated as micromolar (µM) concentration per unit of optical density of the culture at 600 nm, and standard deviations were calculated based on triplicate experiments. HaXAL1, HaXAL2 and FjXAL are the most specific enzymes and those that reach the highest yields.

Table 2. Specific production of pHCA and CA in Escherichia coli (µM OD600-1 +/- standard deviation).

	M9+Tyr	**M9+Tyr**	**M9+Phe**	**M9+Phe**	**M9+His**	**M9+His**
	pHCA	**CA**	**pHCA**	**CA**	**pHCA**	**CA**
No enzyme	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
RmXAL	330 ± 9.7	30 ± 14	9.9 ± 0.3	450 ± 89	27 ± 3.8	35 ± 1
TcXAL	730 ± 23	11 ± 13	22 ± 0.8	510 ± 92		
PcXAL	180 ± 21	2.8 ± 2.7	18 ± 4.9	170 ± 7.7		
RtXAL	170 ± 10	5.9 ± 6.1	7.2 ± 1.3	180 ± 16		
RsTAL	91 ± 13	< 0.05	24 ± 5	4.7 ± 0.4	26 ± 3	0 ± 0
SeSam8	540 ± 50	0 ± 0	76 ± 6	18 ± 5.9	110 ± 26	0 ± 0
FjXAL	440 ± 100	0 ± 0	76 ± 29	0.5 ± 0.4	91 ± 20	0 ± 0
HaXAL1	130 ± 26	0 ± 0	36 ± 14	1.1 ± 0.2		
HaXAL2	61 ± 9.7	0 ± 0	20 ± 4.4	0.4 ± 0.02		

pHCA may be formed from the natural metabolism of *E. coli*, but the production is enhanced by the addition of exogenous tyrosine.

Example 2 – Enzymatic characterization of enzymes

Four of the enzymes were further purified by His-tag purification as follows. A DNA linker (5' phosphorylated oligonucleotides CBJP559 and CBJP560, Table 5) was inserted in place of the sequence between the NdeI and BglII site in plasmid pCDFDuet-1. This would result in the addition of the amino acids MAHHHHHHENLYFQ (SEQ ID NO: 11) to the N-terminal end of the polypeptides. The resulting plasmid was amplified with primers CBJP575 and CBJP576 (table 5) and the genes were amplified and combined using the Gibson reaction (New England Biolabs). The PCR amplification used the same reverse primers as in example 1, but the forward primers matching the His-tag site of the linker (Table 5). Plasmids carrying the genes (e.g. FjXAL, Figure 4) were transformed into electrocompetent

E. coli BL21(DE3)pLysS cells (Life Technologies) and selected on LB plates containing 50 ug/mL streptomycin.

Strains expressing His-tagged versions of the enzymes were grown in LB media overnight at 37°C and diluted into fresh LB media with 1 mM IPTG and growth was propagated overnight (approximately 18 h) at 30°C. Cells were harvested by centrifugation at 8000 rpm for 8 minutes, and disrupted by shearing into a buffer (50 mM Tris-HCl, 10 mM imidazole, 500 mM NaCl, 10% glycerol, pH 7.5). The homogenate was clarified by centrifugation at 10000 g for 10 min at 4°C, and the supernatant was loaded onto Ni2+-NTA resin column on an Äkta Pure system connected to a F9-C fraction collector (GE). Finally the fractions containing the purified polypeptide was dialyzed overnight against a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 10% glycerol, flash-frozen in liquid nitrogen and stored at -80°C.

Enzymatic assays were performed in 200 µL volumes in wells in a UV transparent 96-well plate, by following the increase in absorbance at 315 nm (pHCA) or 295 nm (CA). The reaction mixtures contained 2 µg of purified protein and were initiated by adding 1 mM tyrosine or 6 mM after equilibration to 30°C. The enzymatic activity was calculated as U/g, where U is defined as µmol substrate converted per minute. No conversion was observed in the absence of enzymes under any conditions. Kinetic constants Km and vmax were determined from assays containing 1.56 µM to 200 µM tyrosine or 193 µM to 25 mM phenylalanine.

As table 3 shows, HaXAL1 and FjXAL had the highest catalytic efficiencies (kcat/Km (mM-1 s-1)) towards tyrosine. They also had a very low affinity towards phenylalanine. The most specific enzyme was FjXAL.

Table 3. In vitro kinetics of selected TAL enzymes.

Enzyme	Substrate	Km (µM)	kcat (min-1)	kcat/Km (mM-1 s-1)	TAL/PAL
RsTAL *Rhodobacter sphaeroides*	Tyr	5.6	1.04	3.10	125
	Phe	2400	3.58	0.0246	
SeSam8 *Saccharothrix espanaensis*	Tyr	4.8	0.84	2.93	730
	Phe	2200	0.53	0.00403	
HaXAL1 *Herpetosiphon auranticus*	Tyr	16	3.10	3.29	540
	Phe	22000	7.68	0.00610	
FjXAL *Flavobacterium johnsoniae*	Tyr	5.7	1.27	3.71	3000
	Phe	6600	0.49	0.00123	

Example 3 – Expression of TAL enzymes in *S. cerevisiae*

A number of the previously characterized enzymes were characterized when expressed in *Saccharomyces cerevisiae*. Genes encoding HaXAL1 and FjXAL were synthesized with codon optimization for *S. cerevisiae* available by GeneArt (Life Technologies), and were named HaXAL1Sc and FjXALSc. Genes were amplified using the oligonucleotide (refer to specific name) shown in Table 6, and inserted by uracil-excision into the vector pCfB132 together with the PPGK1 promoter amplified by primers PPGK1_fw and PPGK1_rv (Jensen et al., 2014). The finished plasmids were transformed into *Saccharomyces cerevisiae* CEN.PK102-5B (MATa ura3-**52 his3**Δ**1 leu2**-3/112 MAL2-8c SUC2) using a standard lithium acetate transformation protocol and selected for on synthetic drop-out media plates lacking uracil.

Cells were grown in SC medium without uracil, and diluted into Delft medium or Feed-In-Time (FIT) medium (m2p-labs) supplemented with leucine and histidine. 10 mM tyrosine was added to some cultures as indicated in Table 4. After 72 h of incubation at 30°C with shaking, samples were taken for the analysis of optical density at 600 nm and for clarification of the supernatant, which was analyzed by HPLC as described in example 1. The specific production was calculated as micromolar (μM) concentration per unit of optical density of the culture at 600 nm and is shown in Table 4.

It was evident that HaXAL and FjXAl are the superior enzymes for catalyzing the TAL reaction, while not having background PAL reaction, even when tyrosine is added exogenously.

As demonstrated in Table 4, pHCA may be formed from the natural metabolism of *S. cerevisiae*, but the production may be enhanced by the addition of exogenous tyrosine.

Table 4. Specific production of pHCA and CA in *Saccharomyces cerevisiae* (μM OD600-1 +/- standard deviation).

	Delft		Delft+Tyr		FIT		FIT+Tyr	
Enzyme	**pHCA**	**CA**	**pHCA**	**CA**	**pHCA**	**CA**	**pHCA**	**CA**
PcXAL	46 ± 5.1	17 ± 4.5	200 ± 29	16 ± 5.8	150 ± 65	75 ± 24	200 ± 37	32 ± 14
RtXAL	20 ± 0.8	18 ± 0.9	89 ± 13	21 ± 1.9	67 ± 4.3	88 ± 2	110 ± 8.8	57 ± 4.1
SeSam8	3.1 ± 0.2	0 ± 0	6.9 ± 0.8	0 ± 0	17 ± 1.9	0 ± 0	5.6 ± 1.3	0 ± 0
HaXAL1	31 ± 3.2	0 ± 0	110 ± 15	0 ± 0	140 ± 13	0 ± 0	120 ± 10	0 ± 0
HaXAL1Sc	33 ± 2.6	0 ± 0	140 ± 6.3	0 ± 0	92 ± 16	0 ± 0	160 ± 35	0 ± 0
HaXAL2	22 ± 5.2	0 ± 0	20 ± 6.3	0 ± 0	30 ± 16	0 ± 0	26 ± 11	0 ± 0
FjXAL	30 ± 3.5	0 ± 0	120 ± 16	0 ± 0	130 ± 16	0 ± 0	120 ± 19	0 ± 0
FjXALSc	41 ± 1.6	0 ± 0	150 ± 18	0 ± 0	130 ± 9.7	0 ± 0	200 ± 18	0 ± 0

Table 5. Oligonucleotides used for amplification and synthetic double-stranded DNA

Name	Target	Usage	direction
CBJP483	RsTAL	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2 NdeI
CBJP484	RsTAL	Gibson assembly, Expression in E. coli	reverse Gibson Duet vector MCS2 BglII
CBJP487	RmXAL	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2 NdeI
CBJP488	RmXAL	Gibson assembly, Expression in E. coli	reverse Gibson Duet vector MCS2 BglII
CBJP535	SeSam8	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2 NdeI
CBJP536	SeSam8	Gibson assembly, Expression in E. coli	reverse Gibson Duet vector MCS2 BglII
CBJP553	HaXAL1	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2 NdeI
CBJP554	HAXAL1	Gibson assembly, Expression in E. coli	reverse Gibson Duet vector MCS2 BglII
CBJP555	FjXAL	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2 NdeI
CBJP556	FjXAL	Gibson assembly, Expression in E. coli	reverse Gibson Duet vector MCS2 BglII
CBJP559	Linker for His6 in NdeI+BglII	Restriction Ligation	forward
CBJP560	Linker for His6 in NdeI+BglII	Restriction Ligation	reverse
CBJP561	His-RsTAL	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2::His6 NdeI
CBJP564	His-SeSam8	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2::His6 NdeI
CBJP573	His-HaXAL1	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2::His6 NdeI
CBJP574	His-FjXAL	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2::His6 NdeI
CBJP575	pCDFDuet-1	Gibson assembly, Expression in E. coli	Forward
CBJP576	pCDFDuet-1 modified with His-tag linker	Gibson assembly, Expression in E. coli	Reverse
PPGK1_rv	PGK1 promoter	Uracil Excision, Expression in S. cerevisiae	PG2R
PPGK1_fw	PGK1 promoter	Uracil Excision, Expression in S. cerevisiae	PV2F
CBJP637	SeSam8	Uracil Excision, Expression in S.	GP2F

		cerevisiae	
CBJP638	SeSam8	Uracil Excision, Expression in S. cerevisiae	GV2R
CBJP645	HaXAL1	Uracil Excision, Expression in S. cerevisiae	GP2F
CBJP646	HaXAL1	Uracil Excision, Expression in S. cerevisiae	GV2R
CBJP647	FjXAL	Uracil Excision, Expression in S. cerevisiae	GP2F
CBJP648	FjXAL	Uracil Excision, Expression in S. cerevisiae	GV2R
CBJP649	HaXAL1Sc	Uracil Excision, Expression in S. cerevisiae	GP2F
CBJP650	HaXAL1Sc	Uracil Excision, Expression in S. cerevisiae	GV2R
CBJP651	FjXALSc	Uracil Excision, Expression in S. cerevisiae	GP2F
CBJP652	FjXALSc	Uracil Excision, Expression in S. cerevisiae	GV2R
CBJP741	RtXAL	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2 NdeI
CBJP742	RtXAL	Gibson assembly, Expression in E. coli	reverse Gibson Duet vector MCS2 BglII
CBJP743	TcXAL	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2 NdeI
CBJP744	TcXAL	Gibson assembly, Expression in E. coli	reverse Gibson Duet vector MCS2 BglII
CBJP752	HaXAL2	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2 NdeI
CBJP753	HaXAL2	Gibson assembly, Expression in E. coli	reverse Gibson Duet vector MCS2 BglII
CBJP754	RtXAL	Uracil Excision, Expression in S. cerevisiae	GP2F
CBJP755	RtXAL	Uracil Excision, Expression in S. cerevisiae	GV2R
CBJP762	HaXAL2	Uracil Excision, Expression in S. cerevisiae	GP2F
CBJP763	HaXAL2	Uracil Excision, Expression in S.	GV2R

		cerevisiae	
CBJP812	PcXAL	Gibson assembly, Expression in E. coli	forward Gibson Duet vector MCS2 NdeI
CBJP813	PcXAL	Gibson assembly, Expression in E. coli	reverse Gibson Duet vector MCS2 BglII
CBJP815	PcXAL	Uracil Excision, Expression in S. cerevisiae	forward
CBJP816	PcXAL	Uracil Excision, Expression in S. cerevisiae	reverse

Table 6. Overview of primer pairs used in the Examples

Name	For E. coli Example 1	For His tag Example 2	For S. cerevisiae Example 3
FjXAL	CBJP555 CBJP556	CBJP574 CBJP556	CBJP647 CBJP648
HaXAL1	CBJP553 CBJP554	CBJP573 CBJP554	CBJP645 CBJP646
HaXAL2	CBJP752 CBJP753	-	CBJP762 CBJP763
RsTAL	CBJP483 CBJP484	CBJP561 CBJP484	-
RmXAL	CBJP487 CBJP488	-	-
SeSam8	CBJP535 CBJP536	CBJP564 CBJP536	CBJP637 CBJP638
RtXAL	CBJP741 CBJP742	-	CBJP754 CBJP755
TcXAL	CBJP743 CBJP744	-	-
PcXAL	CBJP812 CBJP813	-	CBJP815 CBJP816
HaXAL1Sc	-	-	CBJP649 CBJP650
FjXALSc	-	-	CBJP651 CBJP652

Example 4 – Expression of TAL enzymes in *Lactococcus lactis*

We have shown that selected TAL enzymes leads to production of *p*-coumaric acid when expressed in *L. lactis*.

The synthetic $RsXAL_{LI}$ (protein GI 129592) and $RmXAL_{LI}$ (protein GI 126464011) genes, codon optimized for *Lactococcus lactis* (GeneArt), were cloned into the nisin inducible expression vector pNZ8048 (Kuipers et al., 1998) as follows: $RsXAL_{LI}$ (SEQ ID NO: 56) and $RmXAL_{LI}$ (SEQ ID NO: 57) genes and the vector were PCR amplified using the primers listed in Table 7, and were assembled in a single-tube isothermal reaction using the Gibson Assembly Master Mix (New England Biolabs). Reaction products were ethanol-precipitated and suspended in double distilled water before transformation into *L. lactis* by electroporation as described by Holo and Nes (1995). The synthetic genes encoding SeSam8, R_XAL, HaXAL1 and FjXAL described in a previous example above were amplified by PCR using the primer pairs listed in Table 1, digested with specific restriction enzymes, and cloned in-between the *Nco*I and *Xba*I restriction sites of pNZ8048. The plasmids were obtained and maintained in *L. lactis* NZ9000 (Kuipers et al., 1998) and the gene sequences of the different constructs were verified by sequencing.

To assess pHCA production, TAL-expression vectors were transformed into a strain derived from NZ9000, but with deletion of the genes *ldh* and *ldhB* (NZ9000Δ*ldh*Δ*ldhB*). A control strain was also constructed by transformation of NZ9000Δ*ldh*Δ*ldhB* with empty expression vector pNZ8048.

For molecular biology procedures, *L. lactis* strains were cultivated as batch cultures (flasks) without aeration in M17 medium (DifcoTM, USA) supplemented with 0.5% glucose (w/v) at 30°C. To assess pHCA production, strains were grown as static cultures in chemically defined medium (CDM; Poolman and Konings, 1988) containing 1% glucose (wt/vol) without pH control (initial pH 6.5 or 7.0) and supplemented with 1.7 or 3.7 mM L-tyrosine. Plasmid selection was achieved by addition of 5 µg mL^{-1} chloramphenicol to the growth medium. Growth was monitored by measuring OD_{600}. For heterologous expression of cloned tyrosine ammonia lyases, *L. lactis* strains were grown in CDM and nisin (1.5 µg L^{-1}) was added at an OD_{600} of 0.3-0.4. Samples (1 mL) of cultures were collected at different points during growth; centrifuged (16,100 × *g*, 10 min, 4°C) and the supernatants stored at -20°C until analysis by HPLC as described in a previous example above. Figure 9 shows the specific *p*-coumaric acid (pHCA) and cinnamic acid (CA) productivities of strains expressing TAL/PAL enzymes in CDM. The first six columns are results from media containing 1.7 mM tyrosine. The seventh and ninth columns represent samples from strains grown in media containing 3.7 mM tyrosine, and the eighth and ninth columns are data from media with 68.5 mM 3-(N-morpholino)propanesulfonic acid (MOPS) and initial pH adjusted to 7.0 rather than 6.5. The strain carrying the empty plasmid ("control") did not result in production of either pHCA or CA under the examined conditions.

Even though the genes encoding $RsXAL_{LI}$ and $RmXAL_{LI}$ had been specifically codon optimized for *L. lactis*, FjXAL showed by far the highest specific production of pHCA (15 µM OD_{600}^{-1}). This corresponds to a five-fold increase in specific production over $RmXAL_{LI}$, the second-best enzyme. The productivities were lower than those achieved in *E. coli*, and the specific productivity of pHCA could be increased (24 µM OD_{600}^{-1}) when the concentration of tyrosine in the media was increased (from 1.7 mM to 3.7 mM) and/or the pH of the medium was increased (from 6.5 to 7.0). $RmXAL_{LI}$ was the only enzyme resulting in production of CA.

Conclusively, the presented TAL enzymes result in specific production of pHCA when expressed in *L. lactis*. Furthermore, the production can be enhanced by manipulation of the supply of the precursor tyrosine and by manipulation of the pH of the growth medium.

Table 7:

Oligonucleotide	**Gene**	**Direction**	**Sequence**	**Restriction site**[a]
LL-Pnis_1			GGTGAGTGCCTCCTTATAATTTATTTTG	
LL-Pnis_2			AAGCTTTCTTTGAACCAAAATTAGAAAACC	
LL-RsXAL-Fw	$RsTAL_{LI}$	Forward	CAAAATAAATTATAAGGAGGCACTCACCATGCTTGCTATGTCACCACCAAAACC	
LL-RsXAL-Rv	$RsTAL_{LI}$	Reverse	GGTTTTCTAATTTTGGTTCAAAGAAAGCTTTTAAACTGGTGATTGTTGTAATAAATG	
LL-RmXAL-Fw	$RmXAL_{LI}$	Forward	CAAAATAAATTATAAGGAGGCACTCACCATGGCTCCATCAGTTGATTCAATTGC	
LL-RmXAL-Rv	$RmXAL_{LI}$	Reverse	GGTTTTCTAATTTTGGTTCAAAGAAAGCTTTTAAGCCATCATTTTAACTAAAACTGG	
LL-SeSam8-Fw	SeSam8	Forward	CATGTCATGACCCAGGTTGTTGAACG	*Bsp*HI
LL-SeSam8-Rv	SeSam8	Reverse	GCTCTAGATTAGCCAAAATCTTTACCATC	*Xba*I
LL-R_XAL-Fw	R_XAL	Forward	GCGGTCTCCCATGCGTAGCGAACAGCTGAC	*Bsa*I
LL-R_XAL-Rv	R_XAL	Reverse	GCTCTAGATTAGGCCAGCAGTTCAATCAG	*Xba*I
LL-HaXAL1-Fw	HaXAL1	Forward	GCGGTCTCCCATGAGCACCACCCTGATTCTG	*Bsa*I
LL-HaXAL1-Rv	HaXAL1	Reverse	GCTCTAGATTAGCGAAACAGAATAATACTACG	*Xba*I
LL-FjXAL-Fw	FjXAL	Forward	CATGTCATGAACACCATCAACGAATATC	*Bsp*HI
LL-FjXAL-Rv	FjXAL	Reverse	GCTCTAGATTAATTGTTAATCAGGTGGTC	*Xba*I

[a] Underlined sequences indicate the respective restriction site.

Example 5 – Production of p-coumaric acid in *Bacillus subtilis*

We have shown that expressing genes encoding tyrosine ammonia-lyases in *Bacillus subtilis* enables production of *p*-coumaric acid, and that the productivity is enhanced when the gene *padC*, encoding a phenolic acid decarboxylase, which is a *p*-coumaric acid degradative enzyme that results in the formation of 4-vinylphenol, is disrupted.

Genes encoding the tyrosine ammonia-lyases SeSam8 and FjXAL were expressed chromosomally in *Bacillus subtilis* as follows. Table 8 lists oligonucleotides used as primers in PCR reactions. A part ("pel end") of the *pel* gen, the region downstream, an erythromycin resistance gene and the constitutive promoter Pcons from *Bacillus subtilis* strain AN214 (US 8,535,911) were PCR amplified using primers CBJP680 and CBJP666. Another part of pel and the region upstream was amplified using primers CBJP667 and CBJP682 ("pel front"). SeSam8 was amplified using primers CBJP689 and CBJP690 and FjXAL was amplified using primers CBJP691 and CBJP692. PCR fragments were combined using splicing by overhang extension PCR (SOE-PCR), with "pel end", "pel front" and either SeSam8 or FjXAL. The two resulting SOE-PCR products were individually integrated into a non-sporulating *Bacillus subtilis* 168 Δ*spoIIAC* deletion strain (Novozymes, US 2011/0306139 A1), selecting for resistance to 5 μg mL^{-1} erythromycin, resulting in strains CBJ1007 and CBJ1008.

The *padC* gene of these strains was furthermore disrupted (inactivated) by integration of a chloramphenicol resistance gene as follows. The chloramphenicol resistance gene of plasmid pC194 (Horinouchi and Weisblum, 1982) was amplified using primers CBJP835 and CBJP836. Regions surrounding padC was amplified using primer pair CBJP837/CBJP838 and CBJP839/CBJP840, respectively. The three fragments were purified from an agarose gel and combined by SOE-PCR. The SOE-PCR product was transformed into CBJ1007 and CBJ1008, and transformants were selected on LB agar plates with 0.2% glucose, 5 μg mL^{-1} erythromycin and 3 μg mL^{-1} chloramphenicol, resulting in strains CBJ1011 and CBJ1012.

To access the productivity, the strains 168 Δ*spoIIAC*, CBJ1007, CBJ1008, CBJ1011 and CBJ1012 were grown in various media. Colonies were used to inoculate growth tubes with 5 mL LB media with 5 μg mL^{-1} erythromycin and 5 μg mL^{-1} chloramphenicol, which were placed shaking at 250 rpm at 37°C overnight before being removed. Samples were withdrawn for HPLC analysis as described in example 1. 10 μL of the cultures in LB media were used to inoculate growth tubes with 5 mL M9 media supplemented with 0.2% glucose

and 50 mg L^{-1} tryptophan with or without 2 mM tyrosine. The tubes were aerated by shaking at 250 rpm at 37°C overnight. Samples were withdrawn for HPLC analysis. *p*-coumaric acid was measured at 333 nm and 4-vinylphenol was measured at 277 nm.

Table 9 shows the productivity as µM pHCA and 4-vinylphenol formed per cell measured at the optical density at 600 nm in a 1-cm light path for three replicates of each experiment. It is evident that the background strain does not produce *p*-coumaric acid and that the productivity reached is higher for the strain expressing FjXAL than SeSam8. Furthermore the productivity is increased in the strains were *padC* is disrupted (inactivated).

Table 8: Oligonucleotides used for PCR reactions

CBJP666	CATGTTTCCTCTCCCTCTCATTTTC
CBJP667	TAAGGTAATAAAAAAACACCTCC
CBJP680	TCATACCATTTTTCACAGGG
CBJP682	GTCTCACTTCCTTACTGCGT
CBJP689	GAAAATGAGAGGGAGAGGAAACATGACCCAGGTTGTTGAACG
CBJP690	GGAGGTGTTTTTTTATTACCTTATCAGCCAAAATCTTTACCATCTGC
CBJP691	GAAAATGAGAGGGAGAGGAAACATGAACACCATCAACGAATATCTG
CBJP692	GGAGGTGTTTTTTTATTACCTTATCAATTGTTAATCAGGTGGTCTTTTACTTTCTG
CBJP835	CCCGCGCGAATATCGTCTGTCCTTCTTCAACTAACGGGGCAG
CBJP836	GAAGTACAGTAAAAGACTAAGGTTATGTTACAGTAATATTGAC
CBJP837	GACGGTTAACTCTGTCACAAGCG
CBJP838	CCTTAGTCTTTTACTGTACTTC
CBJP839	CGGAATCCAATATAGAAGAATGG
CBJP840	GACAGACGATATTCGCGCGGG

Table 9: Productivity of *p*-coumaric acid (pHCA) and 4-vinylphenol (4VP) in *Bacillus subtilis* strains grown in LB media and in M9 medium with 0.2% glucose (M9) or M9 with 2 mM tyrosine.

	LB		M9		M9 with tyrosine	
Genotype	pHCA	4VP	pHCA	4VP	pHCA	4VP
Δ*spoIIAC*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Δ*spoIIAC* *pel*:SeSam8	8.7 ± 0.71	0 ± 0	0 ± 0	0.25 ± 0.021	0 ± 0	0 ± 0
Δ*spoIIAC* *pel*:FjXAL	170 ± 23	1400 ± 200	0 ± 0	65 ± 2.3	36 ± 11	380 ± 37
Δ*spoIIAC* *pel*: SeSam8 Δ*padC*	13 ± 9.1	0 ± 0	0.37 ± 0.047	0 ± 0	2.3 ± 0.44	0 ± 0
Δ*spoIIAC* *pel*:FjXAL Δ*padC*	1000 ± 380	0 ± 0	51 ± 11	0 ± 0	310 ± 26	0 ± 0

Conclusively, *p*-coumaric acid can be produced in *Bacillus subtilis* when expressing a gene encoding a tyrosine ammonia-lyase such as SeSam8 or FjXAL. FjXAL is more efficient in catalyzing this production than SeSam8. A disruption of the gene *padC*, and thereby a degradative pathway, furthermore enhances the productivity and eliminates 4-vinylphenol as a byproduct.

Example 6 – Production of hydroxycinnamic acids other than p-coumaric acid

TAL enzymes have activity toward several aromatic compounds beyond tyrosine. Specifically, we here show that the tyrosine derivatives L-dopa (3,4-dihydroxyphenylalanine or (2S)-2-Amino-3-(3,4-dihydroxyphenyl)propanoic acid) and 3-O-methyldopa (L-3-Methoxytyrosine or 2-Amino-3-(4-hydroxy-3-methoxyphenyl)propanoic acid) are deaminated to caffeic acid and ferulic acid, respectively, by cells expressing selected genes encoding TAL enzymes.

E. coli strains described in Example 1 were used. M9 medium with 0.2 % glucose and 0.5 mM IPTG also containing either tyrosine (204 µM), L-dopa (194 µM) or 3-O-methyldopa (262 µM) was transferred as 3-mL aliquots into a 24-well deep-well plate (Enzyscreen). Aliquots were taken for HPLC before the wells were inoculated with 200 µL of overnight cultures of

the strains. The plates were placed at 37°C with shaking for 16 hours. Samples of the supernatant were withdrawn after two rounds of centrifugation. The samples were subjected to HPLC along with chemical standards as described in Example 1. *p*-coumaric acid, caffeic acid and ferulic acid was measured by absorbance at 333 nm. Tyrosine, L-dopa and 3-O-methyldopa were measured by fluorescence (excitation at 274 nm, emission at 303 nm).

Table 10 shows the concentrations measured from the culture supernatants from duplicate experiments. There was no measurable product in the medium before inoculation.

Table 10: Titers (µM) of *p*-coumaric acid, L-dopa and 3-O-methyldopa in supernatants of *E. coli* cultures expressing different TAL homologs. Cultures were grown in M9 medium with 0.2% glucose (M9) with different additions of substrates as indicated.

Medium	**M9 + tyrosine**	**M9 + L-dopa**	**M9 + 3-O-methyldopa**
Product	***p*-coumaric acid**	**Caffeic acid**	**Ferulic acid**
No Enzyme	0 ± 0	0 ± 0	0 ± 0
SeSam8	102 ± 7.8	5.3 ± 1.1	0.5 ± 0.2
HaXAL1	81 ± 1.2	6.9 ± 1.3	0.6 ± 0.0
FjXAL	215 ± 11.1	5.7 ± 1.0	1.1 ± 0.3

Conclusively, the enzymes HaXAL1 and FjXAL not only catalyze the deamination of tyrosine, but also catalyze the deamination of derivatives thereof. As an example hereof, the enzymes HaXAL1 and FjXAL are shown to use L-dopa and 3-O-methyldopa, which for these particular substrates result in the formation of caffeic acid and ferulic acid. Thus, these enzymes may be used to produce hydroxycinnamic acids using tyrosine or derivatives thereof as substrate.

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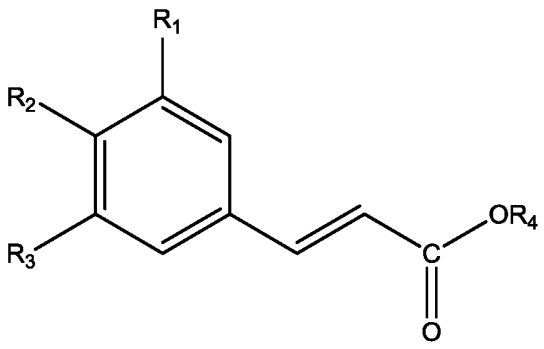
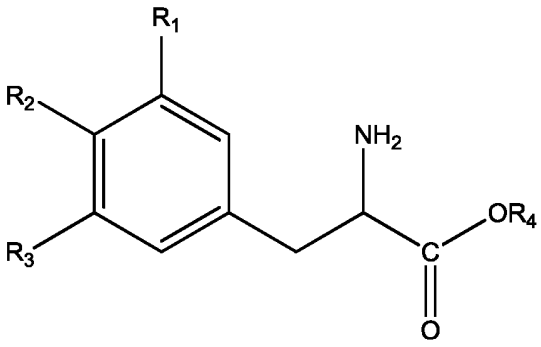
Embodiments of the invention

1. Method for producing a hydroxycinnamic acid of general formula I



general formula I

, the method comprises deaminating a compound of general formula II



general formula II

using a polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3;

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3; or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted;

wherein R_1, R_2 and R_3 independently are selected from the group consisting of hydrogen (H), hydroxyl (-OH), C_{1-6}-alkyl and C_{1-6}-Alkoxy, provided that at least one of R_1, R_2 and R_3 is hydroxyl (-OH); and R_4 is selected from the group consisting of hydrogen (-H) and C_{1-6}-alkyl.

2. The method according to item 1, wherein R_2 is hydroxyl.

3. The method according to item 1 or 2, wherein R_4 is hydrogen.

4. The method according to any one of items 1 to 3, wherein R_1 is hydrogen.

5. The method according any one of items 1 to 4, wherein R_3 is hydrogen or hydroxyl.

6. The method according to any one of items 1 to 5, wherein each of R_1, R_3 and R_4 is hydrogen, and R_2 is hydroxyl.

7. The method according to any one of items 1 to 5, wherein R_1 is hydrogen, R_2 is hydroxyl, R_3 is hydroxyl and R_4 is hydrogen.

8. The method according to any one of items 1 to 7, wherein the polypeptide according to ii) or iii) has tyrosine ammonia lyase activity.

9. The method according to any one of items 1 to 8, wherein the polypeptide according to ii) or iii) comprises the amino acid sequence set forth in SEQ ID NO: 4 or 5.

10. The method according to any one of items 1 to 9, wherein the polypeptide is in isolated form.

11. The method according to item 10, wherein the polypeptide is in purified form.

12. The method according to any one of items 1 to 9, wherein the polypeptide is expressed by a recombinant host cell.

13. The method according to item 12, wherein the recombinant host cell is a microorganism genetically modified to express the polypeptide.

14. The method according to item 12 or 13, wherein the recombinant host cell is selected from the group consisting of bacteria, yeasts, fungi, algae and plant cells.

15. The method according to item 12 or 13, wherein the recombinant host cell is a bacterium.

16. The method according to item 15, wherein the bacterium is a bacterium of the genus *Bacillus, Lactococcus, Lactobacillus, Clostridium, Corynebacterium, Geobacillus,*

Streptococcus, Pseudomonas, Streptomyces, Escherichia, Shigella, Acinetobacter, Citrobacter, Salmonella, Klebsiella, Enterobacter, Erwinia, Kluyvera, Serratia, Cedecea, Morganella, Hafnia, Edwardsiella, Providencia, Proteus, or *Yersinia*.

17. The method according to item 15 or 16, wherein the bacterium is a bacterium of the genus *Bacillus, Lactococcus, Pseudomonas* or *Corynebacterium*.

18. The method according to item 15 or 16, wherein the bacterium is a bacterium of the genus *Bacillus*.

19. The method according to item 18, wherein the bacterium is *Bacillus subtilis*.

20. The method according to item 15 or 16, wherein the bacterium is a bacterium of the genus *Lactococcus*.

21. The method according to item 20, wherein the bacterium is *Lactococcus lactis*.

22. The method according to item 15 or 16, wherein the bacterium is a bacterium of the genus *Pseudomonas*.

23. The method according to item 22, wherein the bacterium is *Pseudomonas putida*.

24. The method according to item 15 or 16, wherein the bacterium is a bacterium of the genus *Corynebacterium*.

25. The method according to item 24, wherein the bacterium is *Corynebacterium glutamicum*.

26. The method according to item 15 or 16, wherein the bacterium is a bacterium of the genus *Escherichia*.

27. The method according to item 26, wherein the bacterium is *Escherichia coli*.

28. The method according to item 12 or 13, wherein the recombinant host cell is a yeast.

29. The method according to item 28, wherein the yeast is of the genus *Saccharomyces, Pichia, Schizosacharomyces, Zygosaccharomyces, Hansenula, Pachyosolen, Kluyveromyces, Debaryomyces, Yarrowia, Candida, Cryptococcus, Komagataella, Lipomyces, Rhodospiridium, Rhodotorula*, or *Trichosporon*.

30. The method according to item 28 or 29, wherein the yeast is a yeast of the genus *Saccharomyces*, or *Pichia*.

31. The method according to any one of items 28 to 30, wherein the yeast is selected from the group consisting of *Saccharomyces cerevisiae, Pichia pastoris*, and *pichia kudriavzevii*.

32. The method according to any one of items 28 to 31, wherein the yeast is *Saccharomyces cerevisiae*.

33. The method according to any one of items 28 to 31, wherein the yeast is *Pichia pastoris*.

34. The method according to item 12 or 13, wherein the recombinant host cell is a fungus.

35. The method according to item 34, wherein the fungus is a fungus of the genus *Aspergillus*.

36. The method according to item 34 or 35, wherein the fungus is *Aspergillus Oryzae* or *Aspergillus niger*.

37. The method according to item 12 or 13, wherein the recombinant host cell is an algae cell.

38. The method according to item 37, wherein the algae cells is an algae cell of the genus *Haematococcus, Phaedactylum, Volvox* or *Dunaliella*.

39. The method according to item 12 or 13, wherein the recombinant host cell is a plant cell.

40. The method according to item 39, wherein the plant cell is selected from the group consisting of soybean, rapeseed, sunflower, cotton, corn, tobacco, alfalfa, wheat, barley, oats, sorghum, lettuce, rice, broccoli, cauliflower, cabbage, parsnips, melons, carrots, celery, parsley, tomatoes, potatoes, strawberries, peanuts, grapes, grass seed crops, sugar beets, sugar cane, beans, peas, rye, flax, hardwood trees, softwood trees, and forage grasses.

41. The method according to any one of items 12 to 40, wherein said recombinant host cell does not express a polypeptide having phenolic acid decarboxylase (PAD) activity.

42. The method according to any one of items 12 to 41, wherein a gene or gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity has been inactivated.

43. The method according to any one of items 12 to 41, wherein said recombinant host cell does not contain within its genome a gene or gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity.

44. A recombinant host cell comprising a heterologous polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3;

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3; or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3, wherein 1 to 50, such as 1 to 40, 1 to 35, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 1 to 3, amino acid residues are substituted, deleted and/or inserted.

45. The method according to item 44, wherein the polypeptide according to ii) or iii) has tyrosine ammonia lyase activity.

46. The method according to item 44 or 45, wherein the polypeptide according to ii) or iii) comprises the amino acid sequence set forth in SEQ ID NO: 4 or 5.

47. The recombinant host cell according to any one of items 44 to 46, the host cell comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding said polypeptide.

48. The recombinant host cell according to item 47, the exogenous nucleic acid molecule further comprises a promoter that is functional in the host cell to cause the production of an mRNA molecule and that is operably linked to the nucleotide sequence encoding said polypeptide.

49. The recombinant host cell according to item 48, the exogenous nucleic acid molecule **further comprises at least one regulatory element selected from a 5' untranslated** region **(5'UTR) and 3' untranslated region (3' UTR).**

50. The recombinant host cell according to any one of items 47 to 49, wherein the exogenous nucleic acid molecule is a vector.

51. The recombinant host cell according to any one of items 47 to 49, wherein the exogenous nucleic acid molecule is stably integrated into the genome of the host cell.

52. The recombinant host cell according to any one of items 44 to 51, wherein the recombinant host cell is selected from the group consisting of bacteria, yeasts, fungi, algae and plant cells.

53. The recombinant host cell according to any one of items 44 to 52, wherein the recombinant host cell is a bacterium.

54. The recombinant host cell according to item 53, wherein the bacterium is a bacterium of the genus *Bacillus, Lactococcus, Lactobacillus, Clostridium, Corynebacterium, Geobacillus, Streptococcus, Pseudomonas, Streptomyces, Escherichia, Shigella, Acinetobacter, Citrobacter, Salmonella, Klebsiella, Enterobacter, Erwinia, Kluyvera, Serratia, Cedecea, Morganella, Hafnia, Edwardsiella, Providencia, Proteus,* or *Yersinia*.

55. The recombinant host cell according to item 53, wherein the bacterium is a bacterium of the genus *Bacillus*.

56. The recombinant host cell according to item 55, wherein the bacterium is *Bacillus subtilis*.

57. The recombinant host cell according to item 53, wherein the bacterium is a bacterium of the genus *Lactococcus*.

58. The recombinant host cell according to item 57, wherein the bacterium is *Lactococcus lactis*.

59. The recombinant host cell according to item 53, wherein the bacterium is a bacterium of the genus *Pseudomonas*.

60. The recombinant host cell according to item 59, wherein the bacterium is *Pseudomonas putida*.

61. The recombinant host cell according to item 53, wherein the bacterium is a bacterium of the genus *Corynebacterium*.

62. The recombinant host cell according to item 61, wherein the bacterium is *Corynebacterium glutamicum*.

63. The recombinant host cell according to item 53, wherein the bacterium is a bacterium of the genus *Escherichia*.

64. The recombinant host cell according to item 63, wherein the bacterium is *Escherichia coli*.

65. The recombinant host cell according to any one of items 44 to 52, wherein the recombinant host cell is a yeast.

66. The recombinant host cell according to item 65, wherein the yeast is of the genus *Saccharomyces, Pichia, Schizosacharomyces, Zygosaccharomyces, Hansenula, Pachyosolen, Kluyveromyces, Debaryomyces, Yarrowia, Candida, Cryptococcus, Komagataella, Lipomyces, Rhodospiridium, Rhodotorula*, or *Trichosporon*.

67. The recombinant host cell according to item 65 or 66, wherein the yeast is a yeast of the genus *Saccharomyces* or *Pichia*.

68. The recombinant host cell according to any one of items 65 to 67, wherein the yeast is selected from the group consisting of *Saccharomyces cerevisiae, Pichia pastoris*, and P*ichia kudriavzevii*.

69. The recombinant host cell according to any one of items 65 to 67, wherein the yeast is *Saccharomyces cerevisiae*.

70. The recombinant host cell according to any one of items 64 to 67, wherein the yeast is *Pichia pastoris*.

71. The recombinant host cell according to any one of items 44 to 52, wherein the recombinant host cell is a fungus.

72. The recombinant host cell according to item 71, wherein the fungus is a fungus of the genus *Aspergillus*.

73. The recombinant host cell according to item 71 or 72, wherein the fungus is *Aspergillus Oryzae* or *Aspergillus niger*.

74. The recombinant host cell according to any one of items 44 to 52, wherein the recombinant host cell is an algae cell.

75. The recombinant host cell according to item 74, wherein the algae cells is an algae cell of the genus *Haematococcus, Phaedactylum, Volvox* or *Dunaliella*.

76. The recombinant host cell according to any one of items 44 to 52, wherein the recombinant host cell is a plant cell.

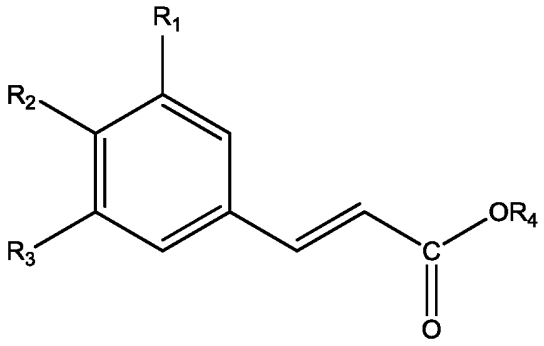
77. The recombinant host cell according to item 76, wherein the plant cell is selected from the group consisting of soybean, rapeseed, sunflower, cotton, corn, tobacco, alfalfa, wheat, barley, oats, sorghum, lettuce, rice, broccoli, cauliflower, cabbage, parsnips, melons, carrots, celery, parsley, tomatoes, potatoes, strawberries, peanuts, grapes, grass seed crops, sugar beets, sugar cane, beans, peas, rye, flax, hardwood trees, softwood trees, and forage grasses.

78. The recombinant host cell according to any one of items 44 to 77, wherein said recombinant host cell does not express a polypeptide having phenolic acid decarboxylase (PAD) activity.

79. The recombinant host cell according to any one of items 44 to 78, wherein a gene or gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity has been inactivated.

80. The recombinant host cell according to any one of items 44 to 78, wherein said recombinant host cell does not contain within its genome a gene or gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity.

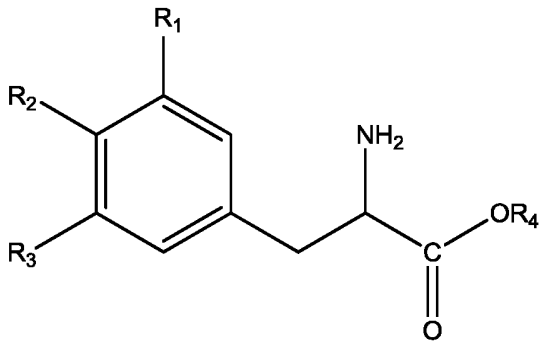
81. A method for producing a hydroxycinnamic acid of general formula I



general formula I

, the method comprises the step of:

a) contacting a recombinant host cell according to any one of items 44 to 80 with a medium comprising a fermentable carbon substrate and/or a compound of the general formula II



general formula II

wherein R_1, R_2 and R_3 independently are selected from the group consisting of hydrogen (H), hydroxyl (-OH), C_{1-6}-alkyl and C_{1-6}-Alkoxy, provided that at least one of R_1, R_2 and R_3 is hydroxyl (-OH); and R_4 is selected from the group consisting of hydrogen (-H) and C_{1-6}-alkyl.

82. The method according to item 81, wherein R_2 is hydroxyl.

83. The method according to item 81 or 82, wherein R_4 is hydrogen.

84. The method according to any one of items 81 to 83, wherein R_1 is hydrogen.

85. The method according any one of items 81 to 84, wherein R_3 is hydrogen or hydroxyl.

86. The method according to any one of items 81 to 85, wherein each of R_1, R_3 and R_4 is hydrogen, and R_2 is hydroxyl.

87. The method according to any one of items 81 to 86, wherein R_1 is hydrogen, R_2 is hydroxyl, R_3 is hydroxyl and R_4 is hydrogen.

88 The method according to any one of items 81 to 87, further comprising the step of:

b) culturing the recombinant host cell under suitable conditions for the production of the hydroxcinnamic acid.

89. The method according to any one of items 81 to 88, further comprising the step of:

c) recovering the hydroxcinnamic acid.

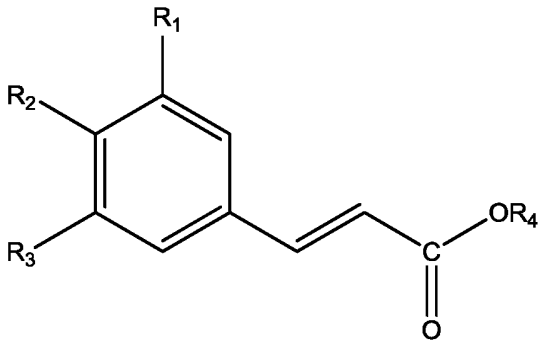
90. Use of a polypeptide in the production of a hydroxycinnamic acid, said polypeptide being selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3;

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3; or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted;

91. The use according to item 90, wherein the hydroxycinnamic acid is of the general formula I



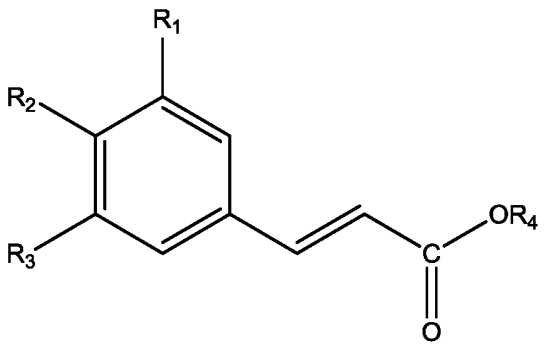
general formula I

wherein R_1, R_2 and R_3 independently are selected from the group consisting of hydrogen (H), hydroxyl (-OH), C_{1-6}-alkyl and C_{1-6}-Alkoxy, provided that at least one of R_1, R_2 and R_3 is hydroxyl (-OH); and R_4 is selected from the group consisting of hydrogen (-H) and C_{1-6}-alkyl.

92. The use according to item 90 or 91, wherein the hydroxycinnamic acid is p-coumaric acid (R_1=H, R_2=OH, R_3=H, R_4=H).

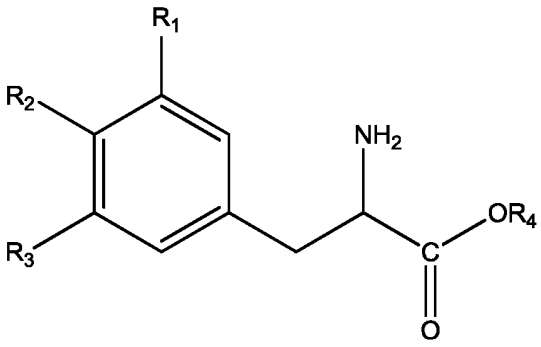
Claims

1. Method for producing a hydroxycinnamic acid of general formula I



general formula I

, the method comprises deaminating a compound of general formula II



general formula II

using a polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3;

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3, wherein the polypeptide has tyrosine ammonia lyase activity; or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are

substituted, deleted and/or inserted, wherein the polypeptide has tyrosine ammonia lyase activity;

wherein R_1, R_2 and R_3 independently are selected from the group consisting of hydrogen (H), hydroxyl (-OH), C_{1-6}-alkyl and C_{1-6}-Alkoxy, provided that at least one of R_1, R_2 and R_3 is hydroxyl (-OH); and R_4 is hydrogen (-H).

2. The method according to claim 1, wherein R_2 is hydroxyl.

3. The method according to claim 1 or 2, wherein each of R_1, R_3 and R_4 is hydrogen, and R_2 is hydroxyl.

4. A recombinant host cell comprising a heterologous polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3;

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3, wherein the polypeptide has tyrosine ammonia lyase activity; or

iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3, wherein 1 to 50, such as 1 to 40, 1 to 35, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 1 to 3, amino acid residues are substituted, deleted and/or inserted, wherein the polypeptide has tyrosine ammonia lyase activity.

5. The recombinant host cell according to claim 4, the host cell comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding said polypeptide.

6. The recombinant host cell according to claim 4 or 5, wherein the recombinant host cell is selected from the group consisting of bacteria, yeasts, fungi, algae and plant cells.

7. The recombinant host cell according to any one of claims 4 to 6, wherein the recombinant host cell is a bacterium.

8. The recombinant host cell according to claim 7, wherein the bacterium is selected from the group consisting of *Escherichia coli*, *Lactococcus lactis, Bacillus subtitlis, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus mojavensis, Streptomyces lividans, Streptomyces griseus, Streptomyces* coelicolor*, Corynebacterium glutamicum,* and *Pseudomonas putida*.

9. The recombinant host cell according to any one of claims 4 to 6, wherein the recombinant host cell is a yeast.

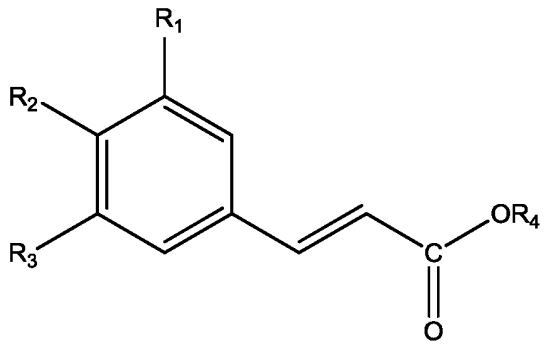
10. The recombinant host cell according to claim 9, wherein the yeast is selected from the group consisting of Saccharomyces cerevisiae, Pichia pastoris, and pichia kudriavzevii.

11. The recombinant host cell according to any one of claims 4 to 10, wherein said recombinant host cell does not express a polypeptide having phenolic acid decarboxylase (PAD) activity.

12. The recombinant host cell according to any one of claims 4 to 11, wherein a gene or gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity has been inactivated.

13. The recombinant host cell according to any one of items 4 to 11, wherein said recombinant host cell does not contain within its genome a gene or gene cluster encoding a polypeptide having phenolic acid decarboxylase (PAD) activity.

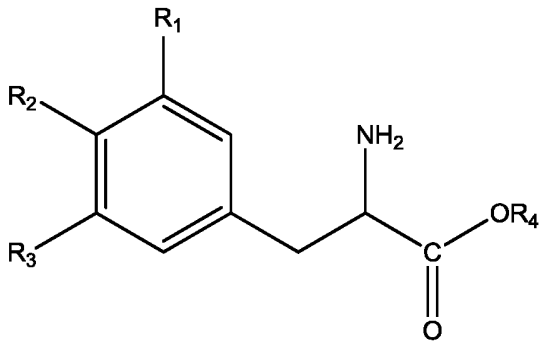
14. A method for producing a hydroxycinnamic acid of general formula I



general formula I

, the method comprises the step of:

a) contacting a recombinant host cell according to any one of claims 4 to 13 with a medium comprising a fermentable carbon substrate and/or a compound of the general formula II



general formula II

wherein R_1, R_2 and R_3 independently are selected from the group consisting of hydrogen (H), hydroxyl (-OH), C_{1-6}-alkyl and C_{1-6}-Alkoxy, provided that at least one of R_1, R_2 and R_3 is hydroxyl (-OH); and R_4 is hydrogen (-H).

15. The method according to claim 14, wherein R_2 is hydroxyl.

16. The method according to claim 14 or 15, wherein each of R_1, R_3 and R_4 is hydrogen, and R_2 is hydroxyl.

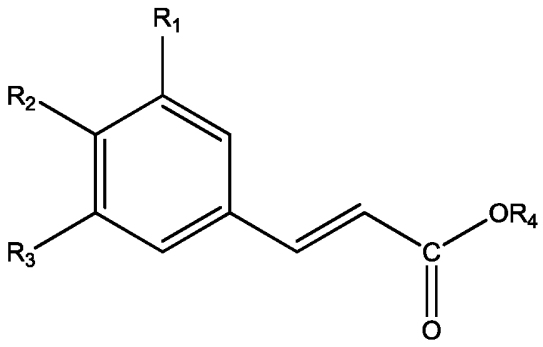
17. Use of a polypeptide in the production of a hydroxycinnamic acid, said polypeptide being selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3;

ii) a polypeptide comprising an amino acid sequence which has at least about 70%, such as at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 1, 2 or 3, wherein the polypeptide has tyrosine ammonia lyase activity; or

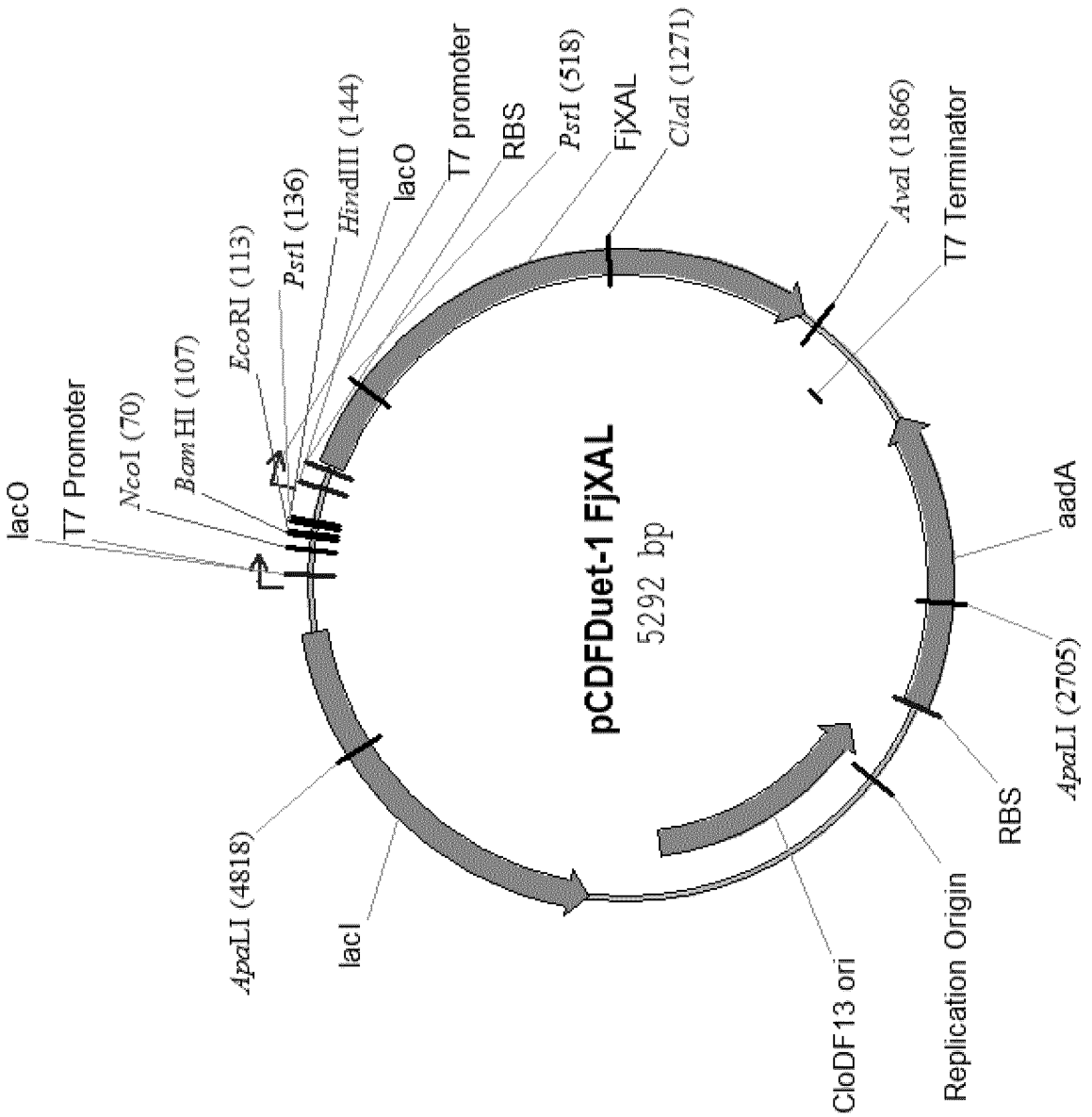
iii) a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 1, 2 or 3, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted and/or inserted, wherein the polypeptide has tyrosine ammonia lyase activity.

18. The use according to claim 17, wherein the hydroxycinnamic acid is of the general formula I



general formula I

wherein R_1, R_2 and R_3 independently are selected from the group consisting of hydrogen (H), hydroxyl (-OH), C_{1-6}-alkyl and C_{1-6}-Alkoxy, provided that at least one of R_1, R_2 and R_3 is hydroxyl (-OH); and R_4 is hydrogen (-H).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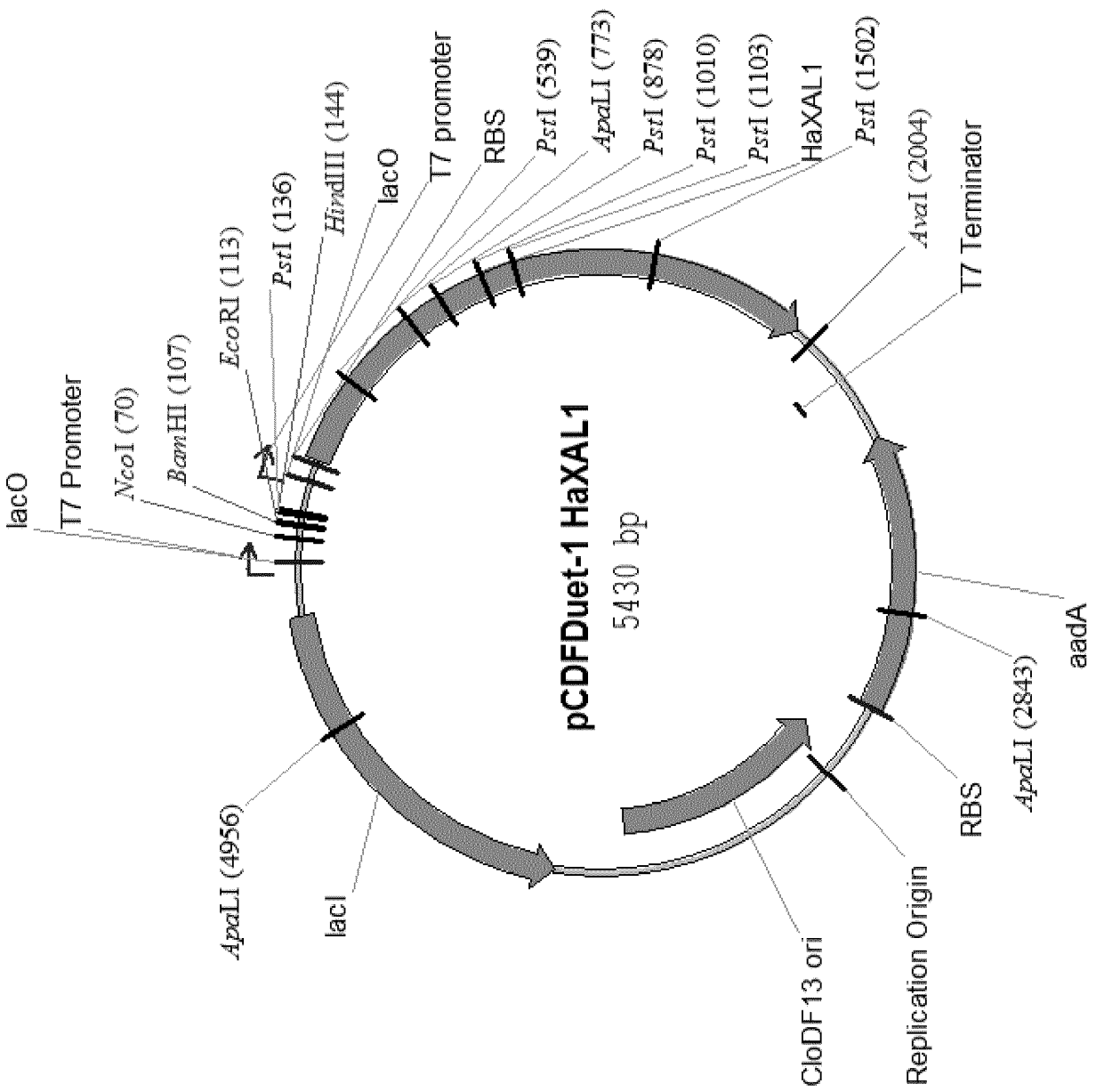
lacO
T7 Promoter
NcoI (70)
BamHI (107)
EcoRI (113)
PstI (136)
HindIII (144)
lacO
T7 promoter
RBS
PstI (518)
FjXAL
ClaI (1271)
AvaI (1866)
T7 Terminator
aadA
ApaLI (2705)
RBS
Replication Origin
CloDF13 ori
lacI
ApaLI (4818)

Figure 1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lacO
T7 Promoter
NcoI (70)
BamHI (107)
EcoRI (113)
PstI (136)
HindIII (144)
lacO
T7 promoter
RBS
PstI (539)
ApaLI (773)
PstI (878)
PstI (1010)
PstI (1103)
HaXAL1
PstI (1502)
AvaI (2004)
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ApaLI (2843)
RBS
Replication Origin
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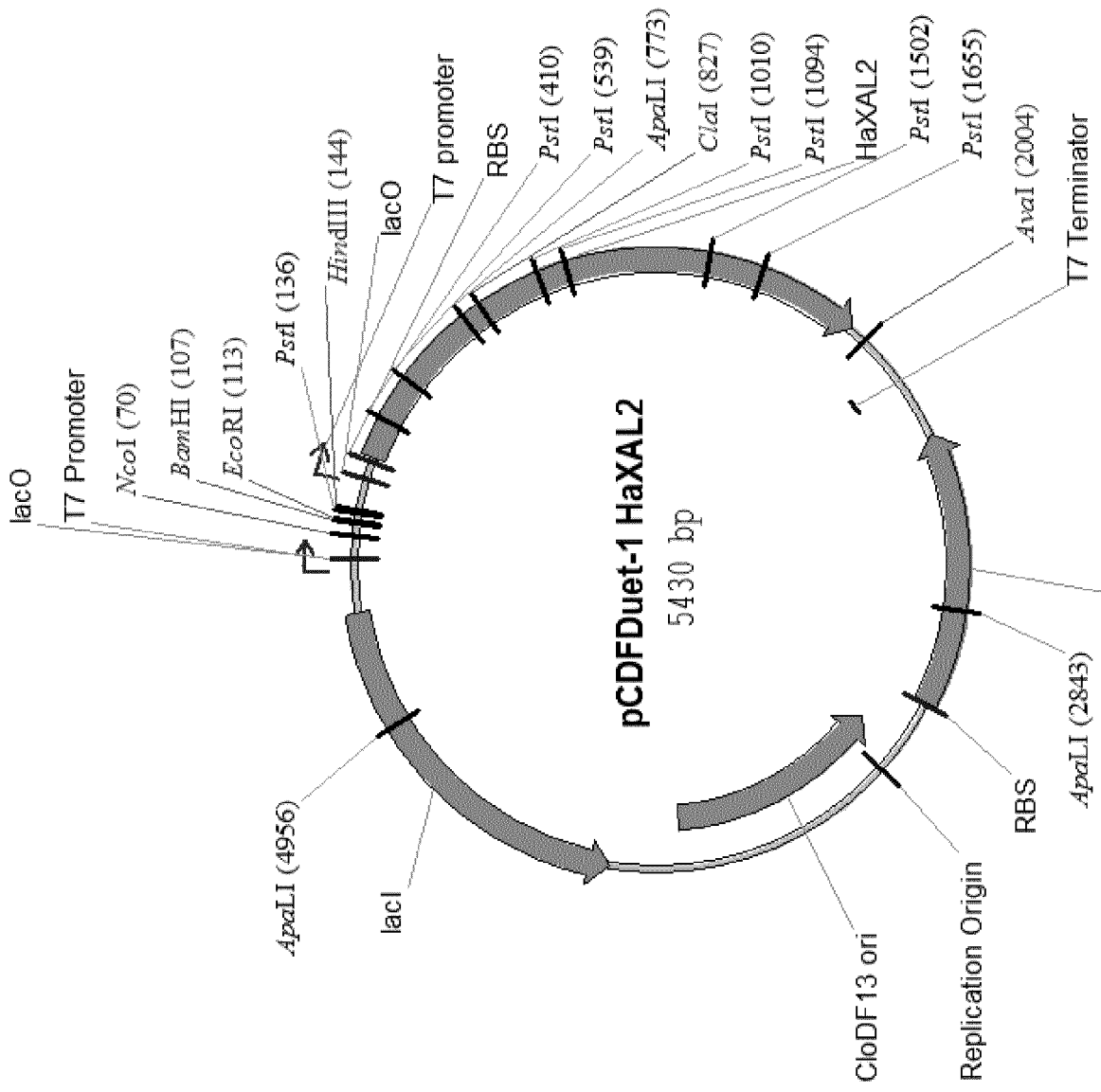
lacI
ApaLI (4956)

Figure 2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lacO
T7 Promoter
NcoI (70)
BamHI (107)
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PstI (136)
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lacO
T7 promoter
RBS
PstI (410)
PstI (539)
ApaLI (773)
ClaI (827)
PstI (1010)
PstI (1094)
HaXAL2
PstI (1502)
PstI (1655)
AvaI (2004)
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aadA
ApaLI (2843)
RBS
Replication Origin
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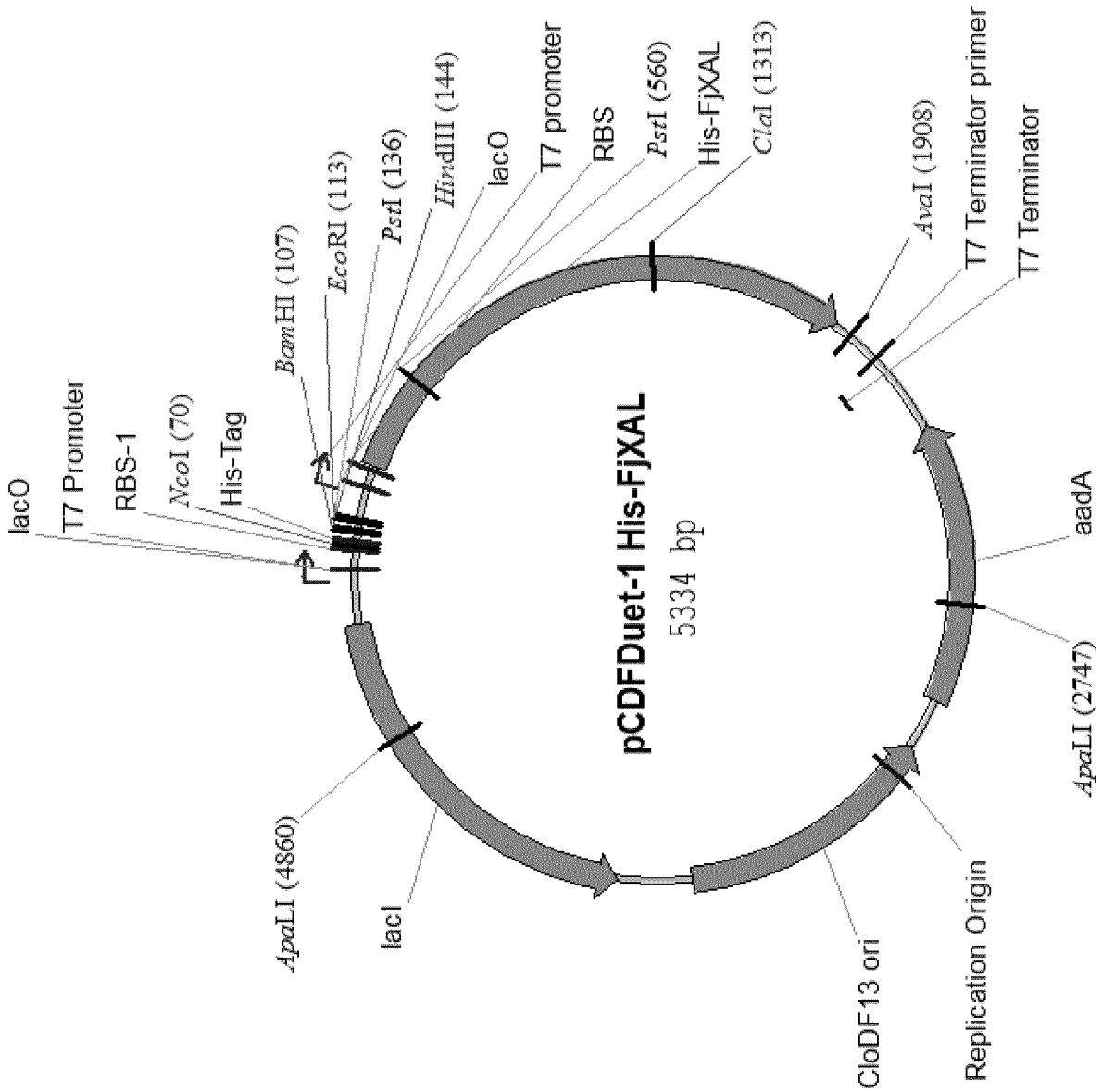
ApaLI (4956)

Figure 3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NcoI (70)
His-Tag
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HindIII (144)
lacO
T7 promoter
RBS
PstI (560)
His-FjXAL
ClaI (1313)
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T7 Terminator
aadA
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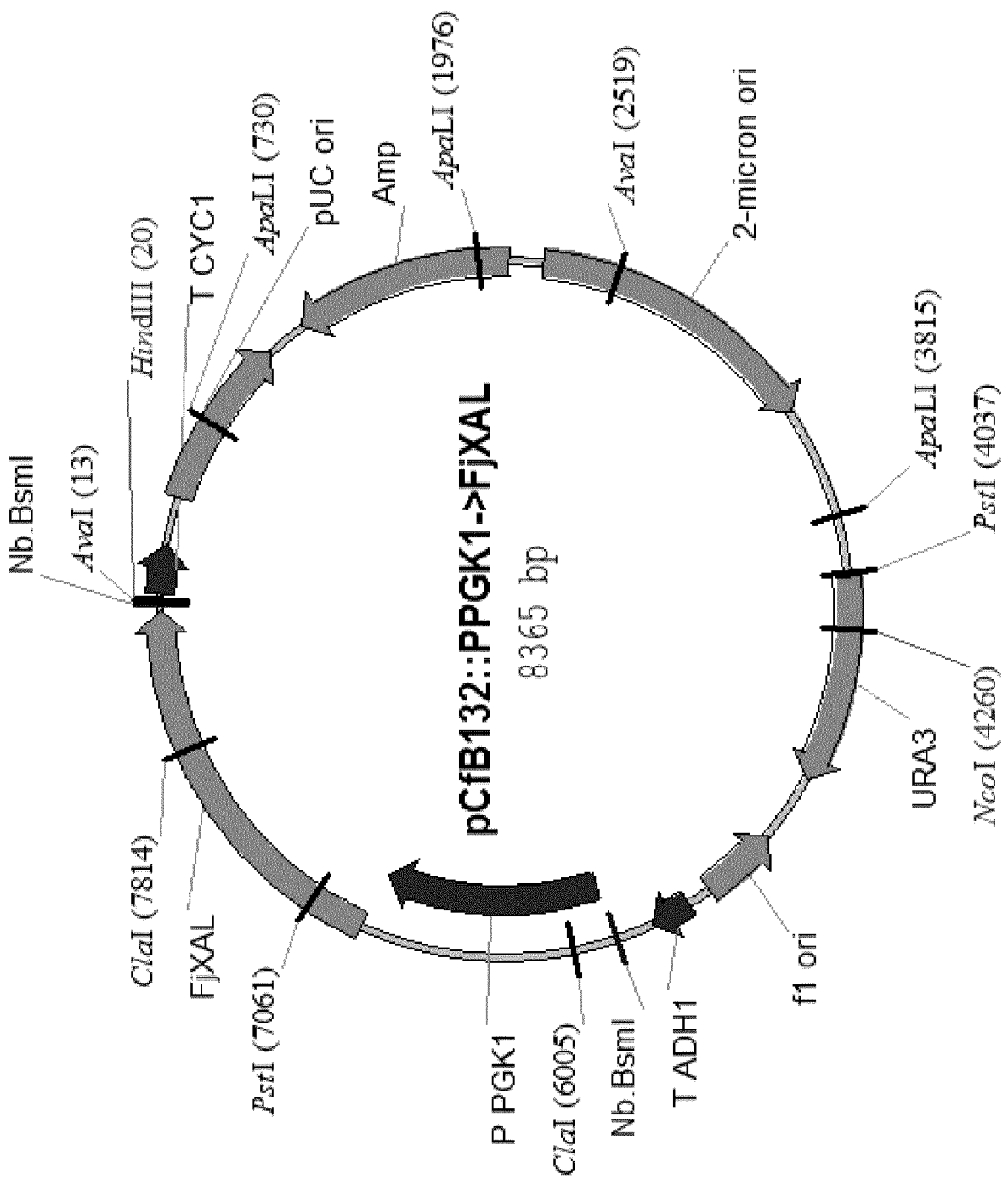
Replication Origin
CloDF13 ori
lacI
ApaLI (4860)

Figure 4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AvaI (13)
HindIII (20)
T CYC1
ApaLI (730)
pUC ori
Amp
ApaLI (1976)
AvaI (2519)
2-micron ori
ApaLI (3815)
PstI (4037)
NcoI (4260)
URA3
f1 ori
T ADH1
Nb.BsmI
ClaI (6005)
P PGK1
PstI (7061)
FjXAL
ClaI (7814)

Figure 5

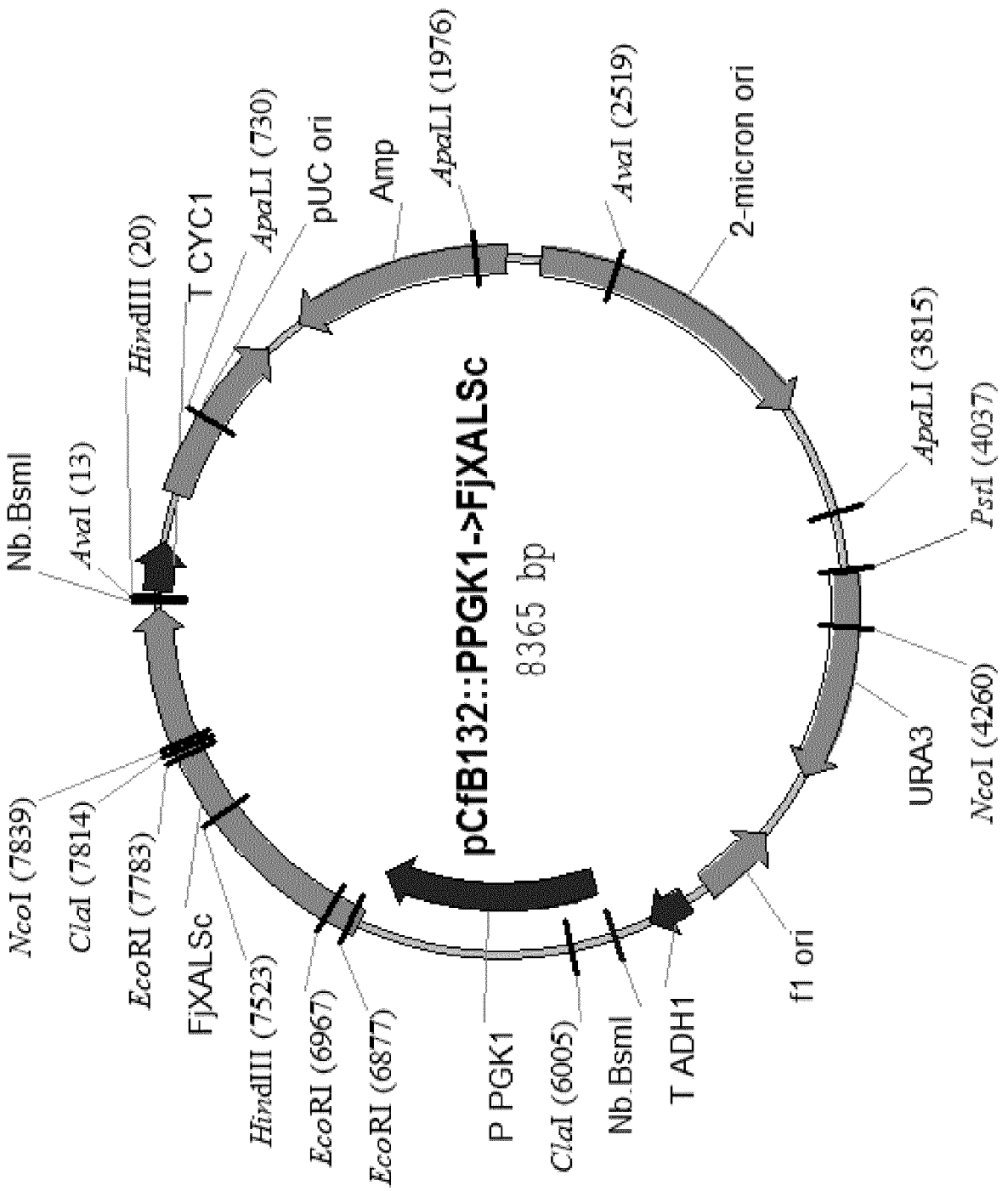
pCfB132::PPGK1->FjXALSc
8365 bp
NcoI (7839)
ClaI (7814)
EcoRI (7783)
FjXALSc
HindIII (7523)
EcoRI (6967)
EcoRI (6877)
P PGK1
ClaI (6005)
Nb.BsmI
T ADH1
f1 ori
URA3
NcoI (4260)
PstI (4037)
ApaLI (3815)
2-micron ori
AvaI (2519)
ApaLI (1976)
Amp
pUC ori
ApaLI (730)
T CYC1
HindIII (20)
AvaI (13)
Nb.BsmI

Figure 6

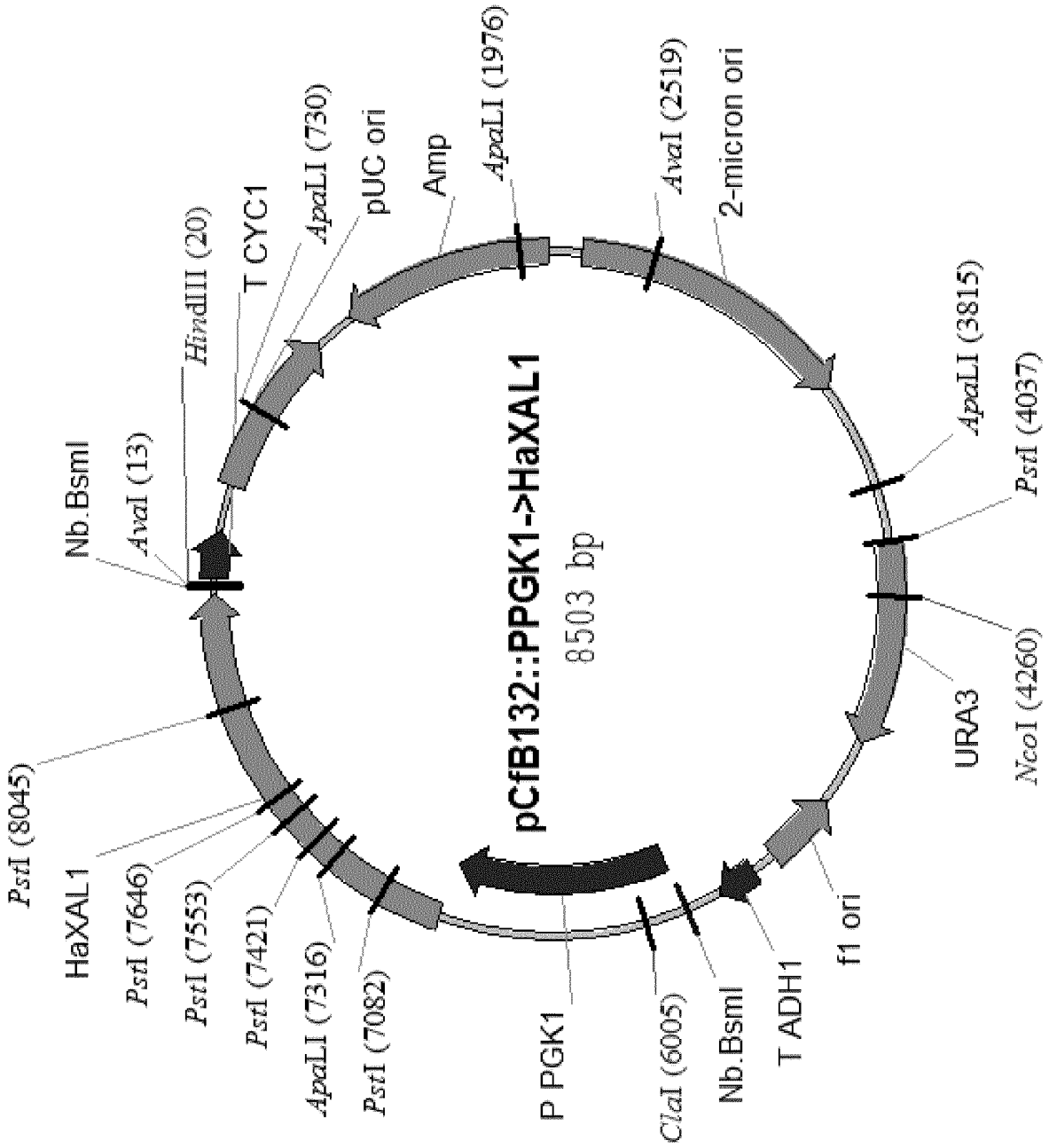
pCfB132::PPGK1->HaXAL1
8503 bp
Nb.BsmI
AvaI (13)
HindIII (20)
T CYC1
ApaLI (730)
pUC ori
Amp
ApaLI (1976)
AvaI (2519)
2-micron ori
ApaLI (3815)
PstI (4037)
NcoI (4260)
URA3
f1 ori
T ADH1
Nb.BsmI
ClaI (6005)
P PGK1
PstI (7082)
ApaLI (7316)
PstI (7421)
PstI (7553)
PstI (7646)
HaXAL1
PstI (8045)

Figure 7

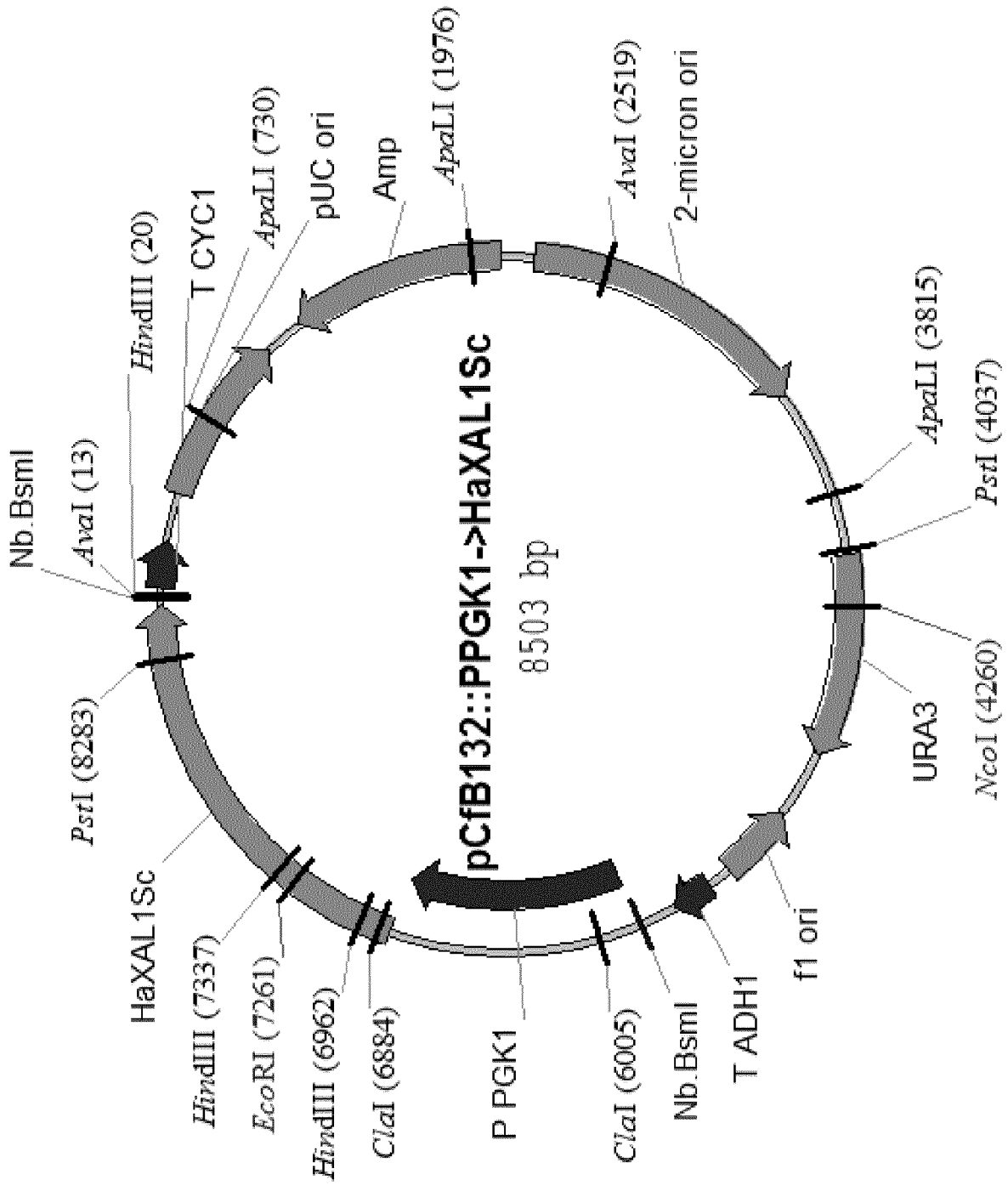
pCfB132::PPGK1->HaXAL1Sc
8503 bp
Nb.BsmI
AvaI (13)
HindIII (20)
T CYC1
ApaLI (730)
pUC ori
Amp
ApaLI (1976)
AvaI (2519)
2-micron ori
ApaLI (3815)
PstI (4037)
NcoI (4260)
URA3
f1 ori
T ADH1
Nb.BsmI
ClaI (6005)
P PGK1
ClaI (6884)
HindIII (6962)
EcoRI (7261)
HindIII (7337)
HaXAL1Sc
PstI (8283)

Figure 8

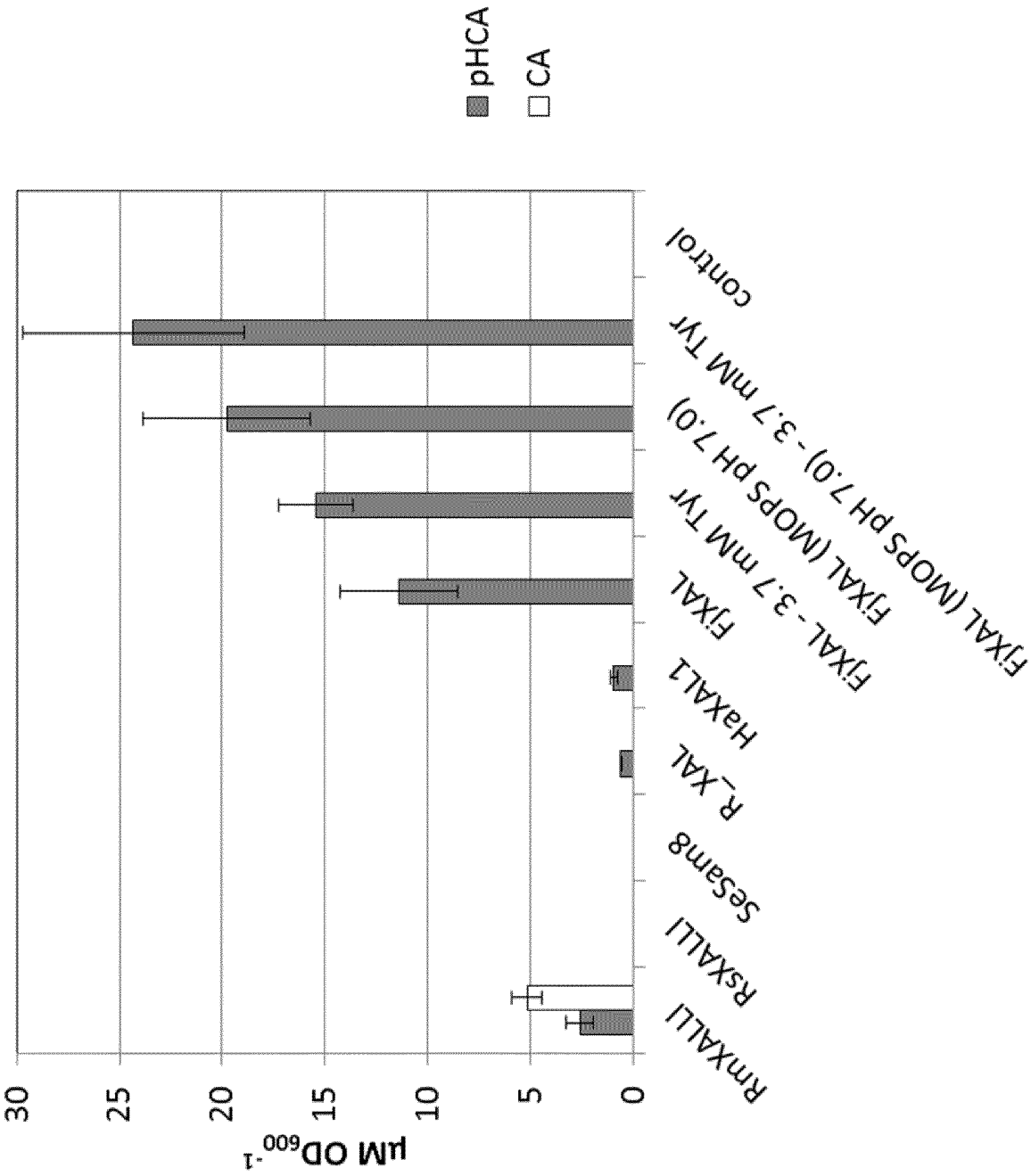
pHCA
CA
μM OD600-1
0
5
10
15
20
25
30
RmXALL
RsXALL
Sesam8
R_XAL
HaXAL1
FjXAL
FjXAL - 3.7 mM Tyr
FjXAL (MOPS pH 7.0)
FjXAL (MOPS pH 7.0) - 3.7 mM Tyr
control

Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2015/066067

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/066067

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/42 C12N9/88
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)
C12P 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 practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, 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	DATABASE UniProt [Online] 12 June 2007 (2007-06-12), "SubName: Full=Histidine ammonia-lyase {ECO:0000313|EMBL:ABQ04142.1}; EC=4.3.1.3 {ECO:0000313|EMBL:ABQ04142.1};", XP002734244, retrieved from EBI accession no. UNIPROT:A5FKY3 Database accession no. A5FKY3 abstract; sequence ----- -/--	4-13

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 September 2015	28/09/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schneider, Patrick

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UniProt [Online] 15 January 2008 (2008-01-15), "SubName: Full=Histidine ammonia-lyase {ECO:0000313¦EMBL:ABX04526.1}; EC=4.3.1.3 {ECO:0000313¦EMBL:ABX04526.1};", XP002734245, retrieved from EBI accession no. UNIPROT:A9AUJ9 Database accession no. A9AUJ9 abstract; sequence	4-13
X	DATABASE UniProt [Online] 15 January 2008 (2008-01-15), "SubName: Full=Histidine ammonia-lyase {ECO:0000313¦EMBL:ABX05046.1}; EC=4.3.1.3 {ECO:0000313¦EMBL:ABX05046.1};", XP002734246, retrieved from EBI accession no. UNIPROT:A9AYR8 Database accession no. A9AYR8 abstract; sequence	4-13
A	US 2005/260724 A1 (BEN-BASSAT ARIE [US] ET AL) 24 November 2005 (2005-11-24) the whole document	1-15
A	QI W W ET AL: "Functional expression of prokaryotic and eukaryotic genes in Escherichia coli for conversion of glucose to p-hydroxystyrene", METABOLIC ENGINEERING, ACADEMIC PRESS, US, vol. 9, no. 3, 1 May 2007 (2007-05-01), pages 268-276, XP025322489, ISSN: 1096-7176, DOI: 10.1016/J.YMBEN.2007.01.002 [retrieved on 2007-05-01] page 269; figure 1	1-15
A	US 7 531 341 B1 (VELLARD MICHEL CLAUDE [US] ET AL) 12 May 2009 (2009-05-12) SEQ 5 is 66% identical to SEQ ID NO:2; abstract; sequence 5	1-15
A	US 2009/047265 A1 (KAKKIS EMIL D [US] ET AL) 19 February 2009 (2009-02-19) Seq 2 has 67% identity with SEQ ID NO3; sequence 2	1-15

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/066067

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	CHRISTIAN BILLE JENDRESEN ET AL: "Highly Active and Specific Tyrosine Ammonia-Lyases from Diverse Origins Enable Enhanced Production of Aromatic Compounds in Bacteria and Saccharomyces cerevisiae", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 81, no. 13, 1 July 2015 (2015-07-01), pages 4458-4476, XP055214411, ISSN: 0099-2240, DOI: 10.1128/AEM.00405-15 the whole document -----	1-18

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/066067

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