Abstract: The present invention relates to uses of genetically modified recombinant micro-organisms to produce styrene from carbon substrates or to convert \( \text{trans} \)-cinnamic acid into styrene. Especially the invention relates to microbiological methods for producing styrene or for converting \( \text{trans} \)-cinnamic acid into styrene. The invention further relates to a recombinant fungus that has been genetically modified and which can be utilized in said processes.
METHODS FOR PRODUCING STYRENE AND GENETICALLY MODIFIED MICRO-ORGANISMS RELATED THERETO

FIELD OF THE INVENTION

The present invention relates to uses of genetically modified recombinant micro-organisms to produce styrene from carbon substrates or to convert trans-cinnamic acid into styrene. Especially the invention relates to microbiological methods for producing styrene or for converting trans-cinnamic acid into styrene. The invention further relates to a recombinant fungus that has been genetically modified and which can be utilized in said processes.

BACKGROUND OF THE INVENTION

Styrene is a versatile commodity chemical which is mainly used to make polymers and co-polymers. It is currently produced exclusively from fossil petroleum, but as oil stocks become depleted and environmental issues are more emphasized it will be increasingly important to replace petroleum-derived styrene with styrene from alternative sources.

When styrene is to be produced from renewable resources a pathway from L-phenylalanine through trans-cinnamic acid to styrene has been suggested. It has been described that a bacterium can be engineered to convert glucose to styrene. In such an organism D-glucose is converted to L-phenylalanine and L-phenylalanine is further converted in two steps first to trans-cinnamic acid by a phenylalanine ammonia lyase, PAL, and then to styrene by a cinnamic acid decarboxylase (see Figure 1) (McKenna and Nielsen, 2011, Metab Eng. 13, 544-554).

Enzymes for the decarboxylation of cinnamic acids have been described in various micro-organisms such as bacteria, yeasts and molds.

In the yeast Saccharomyces cerevisiae the PAD1 gene (phenylacrylate decarboxylase gene 1) has been identified as a gene that confers resistance toward cinnamic acid (Clausen et al. 1994, Gene 142, 107-112). The deletion of PAD1 results in the inability to convert cinnamic acid to styrene (Stratford et al. 2007, Appl Environ Microbiol. 73, 6534-6542) and the overexpression of the PAD1 gene results in strains with increased resistance to cinnamic and ferulic acid (Larsson et al. 2001, Appl. Microbiol. Biotechnol. 57 (2001) 167-174).

The FDC1 gene (ferulic acid decarboxylase gene 1) has also been identified in the yeast Saccharomyces cerevisiae. However, the role of the
**FDC1** alone for the decarboxylation activity is not shown unambiguously (Mukai et al. 2010, J. Biosci. Bioeng. 109, 564-569).

Styrene production is not always desired. This is the case for example in food industry, wherein decarboxylation reactions result in off-flavors. There is a study wherein off-flavors are tried to be prevented in the production of alcoholic beverages (Mukai et al. 2010, J. Biosci. Bioeng. 109, 564-569). Because decarboxylation of cinnamic acid, ferulic acid or coumaric acid is crucial for the production of unpleasant flavors, inhibition of these activities by genetic modifications was studied. Deletions in the **PAD1** and/or the **FDC1** gene resulted in a reduced ability to decarboxylate cinnamic acid, ferulic acid and coumaric acid. The decarboxylation activity was recovered when the deleted genes **PAD1** and/or **FDC1** were retransformed. The authors suggest that the decarboxylation of ferulic acid in yeast can be increased by overexpression of **FDC1**.

Contrary to Mukai et al., the goal of WO2012122333 A1 is to produce styrene, and thus to support the production of polymers and co-polymers. WO2012122333 A1 describes a method for the production of styrene by utilizing at least one gene encoding a polypeptide having trans-cinnamic acid decarboxylase activity. **FDC1** is mentioned as a suitable gene for this decarboxylase activity.

Also McKenna and Nielsen intend to produce styrene (McKenna and Nielsen, 2011, Metab Eng. 13, 544-554). To express trans-cinnamic acid decarboxylase activity in *E. coli* whole cells they utilized S. cerevisiae **PAD1** and **FDC1** genes. The expression of the S. cerevisiae **FDC1** alone resulted in cinnamic acid decarboxylation activity in *E. coli* but the expression of **PAD1** alone did not, suggesting that **PAD1** is not required for such activity. The results demonstrate that **FDC1** expression is sufficient for the decarboxylation of trans-cinnamic acid to styrene. **PAD1** co-expression with **FDC1** is not needed for the decarboxylation of trans-cinnamic acid and neither increases nor alters trans-cinnamic acid decarboxylase activity in *E. coli*. The styrene production was also hampered by the high toxicity of styrene toward *E. coli*.

In summary, a pathway for styrene production with decarboxylases has been described but the conversion of cinnamic acid to styrene occurred at a very slow rate, and the amount of styrene obtained was very low due to its toxicity. The present invention aims at overcoming these disadvantages, and
improving the biological production of styrene. One aim of the present invention is to produce styrene more efficiently and faster.

BRIEF DESCRIPTION OF THE INVENTION

An object of the present invention is to provide a low cost process for large scale biological production of styrene by specific genetically modified micro-organisms. The objects of the invention are achieved by a method and an arrangement, which are characterized by what is stated in the independent claims. Preferred embodiments of the invention are disclosed in the dependent claims.

Efficient enzymes and the corresponding encoding genes are presented herein. The present invention is based on identifying genes which, when over-expressed in the micro-organisms, provide advantageous tools for mass production of styrene and furthermore, for polymer industries. We have surprisingly found that the genes PAD1 and FDC1 have to be expressed simultaneously in a genetically engineered organism, in order to produce high yields of styrene with high rates. This is the key to production of styrene from biomass.

We have also surprisingly found a genetically modified microorganism, preferably a fungus, which is resistant to styrene. In the micro-organisms of the present invention, achievable styrene titers are not toxic and thus, production of styrene in high amounts is possible. Styrene is preferably recovered directly from the culture medium. Styrene has a low solubility in water which facilitates the downstream processing.

The present invention relates to a method of producing styrene, said method comprising

a) providing a recombinant micro-organism that has been genetically modified to overexpress the PAD1 and FDC1 genes,

b) culturing said recombinant micro-organism in a carbon substrate containing medium to obtain styrene, and

c) recovering the resulting styrene from the medium.

Also, the present invention relates to a method of converting transcinnamic acid to styrene, said method comprising

a) providing a recombinant micro-organism that has been genetically modified to overexpress the PAD1 and FDC1 genes,
b) culturing said recombinant micro-organism in a trans-cinnamic acid containing medium to obtain styrene, and

c) recovering the resulting styrene from the medium.

Further, the present invention relates to the use of a recombinant micro-organism that has been genetically modified to overexpress the PAD1 and FDC1 genes for producing styrene of a carbon substrate or for converting trans-cinnamic acid to styrene.

Still, the present invention relates to a recombinant fungus that has been genetically modified to overexpress the PAD1 and FDC1 genes.

BRIEF DESCRIPTION OF THE DRAWINGS

In the following the invention will be described in greater detail by means of preferred embodiments with reference to the attached drawings, in which

Figure 1 shows a reaction scheme for styrene production from L-phenylalanine. L-phenylalanine can be produced from renewable sugars e.g. glucose;

Figure 2 shows the cinnamic acid concentration after addition of S. cerevisiae cell extract overexpressing S. cerevisiae FDC1 and PAD1 (squares), only FDC1 (circles) or only PAD1 (triangles). The protein concentration is about 0.2 mg/ml;

Figure 3 shows the cinnamic acid concentration after addition of E. coli cell extract overexpressing S. cerevisiae FDC1 and PAD1 (triangles), only FDC1 (circles) or only PAD1 (squares);

Figure 4A shows growth of the S. cerevisiae strain overexpressing PAL, FDC1 and PAD1 with strong constitutive promoters integrated to the chromosomes in the presence of varying concentrations of 4-fluoro-phenylalanine;

Figure 4B shows growth of the control S. cerevisiae strain in the presence of varying concentrations of 4-fluoro-phenylalanine.

DETAILED DESCRIPTION OF THE INVENTION

A method was identified to generate micro-organisms that can efficiently convert cinnamic acid to styrene. This is the key step to engineer micro-organisms for the conversion of biomass to styrene. Any micro-organisms or strains thereof insensitive to styrene are suitable according to the present invention.
The present invention identifies a favorable enzyme activity and a genetically modified micro-organism for efficient decarboxylation of cinnamic acid to styrene. Embodiments of the present invention point out metabolically engineered microbial strains for the production of styrene.

Production of large amounts of styrene is a challenge. In microbial production a good source of phenylalanine is needed for converting to cinnamic acid and further to styrene (Figure 1). In the pathway of styrene production (Figure 1) first L-phenylalanine is deaminated by a phenylalanine ammonia lyase, PAL, to produce trans-cinnamic acid. The PAL enzyme activity has been previously described; the corresponding gene has been identified from several organisms and the expression of an active enzyme in different heterologous hosts has been described previously (Faulknera et al. 1994, Gene 143, 13-20). Second, trans-cinnamic acid is decarboxylated by a decarboxylase to form styrene (McKenna and Nielsen, 2011, Metab Eng. 13, 544-554).

In the present invention we were able to show cinnamic acid decarboxylase activity in cell extracts of micro-organisms, when mixing extracts where the PAD1 and the FDC1 were expressed. In Examples 1-4 we show that the overexpression of the PAD1 and the FDC1 results in an increased rate of trans-cinnamic acid decarboxylation activity in genetically modified crude cell extracts. The overexpression of PAD1 or FDC1 alone did not result in increased activity.

The increased decarboxylase activity is also present in intact cells as it is shown in Example 1 by a higher tolerance toward trans-cinnamic acid. This was tested by following the growth on plates with different concentrations of trans-cinnamic acid. The parent strain, as well as strains expressing only PAD1 or FDC1 showed reduced growth at 500 µM and 1 mM cinnamic acid but no growth at higher concentrations. The strain expressing PAD1 and FDC1 was growing up to concentrations of 10 mM trans-cinnamic acid. (Examples 1, 2, 4)

**PAD1 and FDC1**

Many enzymes for decarboxylation of hydroxycinnamic acid, such as ferulic acid, P-coumaric acid and caffeininc acid have been described before. Hydroxycinnamic acid decarboxylation enzymes are known for example in bacteria, yeasts and molds.
A ferulic acid decarboxylase and the corresponding gene, \textit{fdc}, was identified from \textit{Bacillus pumilus} (Zago et al., 1995, Appl Environ Microbiol 61, 4484-86). A ferulic acid decarboxylase was also identified from an \textit{Enterobacter} species (Gu et al., 201 1a, Appl Microbiol Biotechnol 89, 1797-1 805) and a crystal structure obtained (Gu et al., 201 1b, PLoS One 6 e16262).

A p-coumaric acid decarboxylase was purified from \textit{Lactobacillus plantarum}. The enzyme was inducible and the purified enzyme had a $K_M$ of 1.4 mM and a $K_{cat}$ of $10^3$ s$^{-1}$ (Cavin et al., 1997b, FEMS Microbiol Lett 147, 291-295). The corresponding gene, \textit{pdc}, was cloned and overexpressed in \textit{E. coli} (Cavin et al., 1997a, Appl Environ Microbiol 63, 1939-1 944). From this protein also a crystal structure was obtained (Rodriguez et al., 2010, Proteins 78, 1662-1 676).

Based on the homology to the \textit{fdc} and \textit{pdc} a hydroxycinnamic acid (phenolic acid) decarboxylase, \textit{pad}, was identified in \textit{Bacillus subtilis} (Cavin et al. 1998, Appl Environ Microbiol 64, 1466-1471 ). The \textit{pdc} was used to engineer an \textit{E. coli} strain to produce p-hydroxy styrene. The strain also contained a tyrosine ammonia lyase (TAL) to convert tyrosine to p-coumaric acid, the substrate for the PDC (Qi et al. 2007, Metab Eng 9, 268-276).

The hydroxycinnamic acid decarboxylases \textit{fdc}, \textit{pdc} and \textit{pad} have been shown to be active \textit{in vivo} and the purified enzymes do not require cofactors and are not part of multi subunit enzyme complexes, however none of these enzymes have shown activity with trans-cinnamic acid.

Also enzymes for decarboxylation of cinnamic acid or \textit{trans-}cinnamic acid have been described before. The yeast \textit{Saccharomyces cerevisiae} shows some resistance toward cinnamic acid and the gene that confers this resistance was identified as the \textit{PAD1} gene (Clausen et al. 1994, Gene 142, 107-1 12). A \textit{PAD1} homologue is also present in the mold \textit{Aspergillus niger} where it confers resistance to cinnamic acid (Plumridge et al. 2008, Fungal Genetics and Biology 47, 683-692). The association of the \textit{FDC1} with cinnamic acid resistance has also been shown in \textit{Saccharomyces cerevisiae} (Mukai et al. 2010, J. Biosci. Bioeng. 109, 564-569).

The \textit{PAD1} and the \textit{FDC1} genes are clustered, i.e. they are located next to each other on the chromosomes. Such clusters with \textit{PAD1} and \textit{FDC1} homologues are widespread in yeast and filamentous fungi. In filamentous fungi the cluster contains also a transcription factor (Plumridge et al. 2010, Fungal Genetics and Biology 47, 683-692).
The Pad1 and the Fdc1 proteins are believed to be in different cellular compartments. The PAD1 has mitochondrial targeting sequence and the protein is located in the mitochondria (Huh et al. 2003, Nature Chemical Biology 425, 686-691) whereas the FDC1 is a cytosolic protein.

As used herein "PAD1" refers to the phenylacrylate decarboxylase

1. "PAD1" refers to not only S. cerevisiae PAD1 but also PAD1 homologues from any micro-organisms. As an example, the enzyme encoded by the S. cerevisiae PAD1 gene is described for example in the article of Clausen M et al. (1994, Gene 142(1): 107-12).

As used herein "FDC1" refers to the ferulic acid decarboxylase 1.

2. "FDC1" refers to not only S. cerevisiae FDC1 but also FDC1 homologues from any micro-organisms. As an example, the enzyme encoded by the S. cerevisiae FDC1 gene is described for example in the article of Mukai N et al. (J. Biosci. Bioeng. 109 (2010) 564-569) and http://www.yeastgenome.org/cgi-bin/locus.fp?locus=FDC1.

As used herein "PAL" refers to the phenylalanine ammonia lyase. "PAL" refers to not only S. cerevisiae PAL but also PAL homologues from any micro-organisms. The enzyme encoded by the PAL gene is classified as EC 4.3.1.24.

According to the present invention it is not required to express the PAD1 and/or FDC1 from S. cerevisiae. Homologues of PAD1 and FDC1 from other micro-organisms can also be used. Furthermore, PAD1 and FDC1 genes from different organisms can be combined for the micro-organism of the present invention. For example we tested an FDC1 homologue from Aspergillus niger together with the PAD1 from S. cerevisiae and observed the same decarboxylase activity (Example 2). Homologues of S. cerevisiae PAD1 and FDC1 can be identified by any conventional methods known in the art, for example by carrying out a BLAST or FASTA search with the translated proteins of an organism to identify the proteins with the highest homology to S. cerevisiae PAD1 or FDC1.

In one embodiment of the invention at least one of the PAD1 and FDC1 genes is derived from a yeast, a filamentous fungus, or a bacterium. Therefore, the following combinations are possible: PAD1 and FDC1 derived from a yeast; PAD1 and FDC1 derived from a filamentous fungus; PAD1 and FDC1 derived from a bacterium; PAD1 derived from a yeast and FDC1 derived from a filamentous fungus; PAD1 derived from a yeast and FDC1 derived from
a bacterium; PAD1 derived from a filamentous fungus and FDC1 derived from a yeast; PAD1 derived from a filamentous fungus and FDC1 derived from a bacterium; PAD1 derived from a bacterium and FDC1 derived from a yeast; PAD1 derived from a bacterium and FDC1 derived from a filamentous fungus.

In a specific embodiment of the invention at least one of the PAD1 and FDC1 genes is derived from *Saccharomyces cerevisiae, E. coli,* or *Aspergillus niger* i.e. *S. cerevisiae* PAD1 and FDC1; *E. coli* PAD1 and FDC1; *Aspergillus niger* PAD1 and FDC1; *S. cerevisiae* PAD1 and *E. coli* FDC1; *S. cerevisiae* PAD1 and *Aspergillus niger* FDC1; *E. coli* PAD1 and *S. cerevisiae* FDC1; *E. coli* PAD1 and *Aspergillus niger* FDC1; *Aspergillus niger* PAD1 and *S. cerevisiae* FDC1; *Aspergillus niger* PAD1 and *E. coli* FDC1. "Derived from" in this connection means that the gene may be isolated from said microorganism source and optionally further modified. It may also be a synthetic version, where the codon usage may be optimized for expression in the micro-organism in question. The term does not exclude minor modifications of the sequence e.g. by substitution, deletion, and/or insertion of one or a few nucleotides as long as the enzymatic activity of the encoded protein is retained.

The amino acid sequence for the *S. cerevisiae* PAD1 is identified as SEQ ID NO:15, the amino acid sequence for the *Aspergillus niger* PAD1 is identified as SEQ ID NO:17, the amino acid sequence for the *S. cerevisiae* FDC1 gene is identified as SEQ ID NO:16, and the amino acid sequence for the *Aspergillus niger* FDC1 is identified as SEQ ID NO:18.

PAD1 or FDC1 suitable for the present invention include those that have at least 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with amino acid sequences of SEQ ID NO:s 15, 16, 17 or 18. Identity of any sequence compared to the PAD1 or FDC1 sequence of this invention refers to the identity of any sequence compared to the entire PAD1 or FDC1 sequence of the present invention. Sequence identity may be determined for example by using BLAST (Basic Local Alignment Search Tools) or FASTA (FAST-AII). In the searches, setting parameters "gap penalties" and "matrix" are typically selected as default.
Any polynucleotide sequences encoding amino acids of PAD1 or FDC1, for example as described in any of SEQ ID NO:s 15-18, or encoding any parts thereof having function of PAD1 or FDC1 can be used in the present invention.

5 Genetic modifications

The genetically modified micro-organisms used in the invention are made by performing genetic modifications to a micro-organism cell. As used herein "a recombinant micro-organism" refers to any micro-organism that has been genetically modified to contain different genetic material compared to the micro-organism before modification. The micro-organism of the invention is genetically modified to produce styrene by overexpression of the PAD1 and FDC1 genes. "The recombinant micro-organism" of the invention also refers to a host cell comprising a vector or plasmid comprising polynucleotides comprising the PAD1 and FDC1 genes.

A recombinant micro-organism that has been "genetically modified to overexpress" includes embodiments, where a protein-encoding polynucleotide has been transformed into a cell in such a manner that the cell is capable of producing an active protein. As used herein, "overexpression" achieved by genetic modifications of a micro-organism refers to excessive expression of a gene by producing more products (e.g. protein) than an unmodified micro-organism. One or more copies of a gene or genes may be transformed to a cell for overexpression. The term also encompasses embodiments, where a promoter region has been modified to allow the expression of the gene or a homologous gene in a micro-organism. A promoter region may also be modified for more effective expression of a gene. The micro-organisms or the fungi of the present invention overexpress at least the PAD1 and the FDC1 genes. In one embodiment of the invention the micro-organisms or the fungi overexpress only the PAD1 and the FDC1 genes.

In one embodiment of the invention the recombinant micro-organism or fungus further comprises other genetic modifications than the overexpression of the PAD and FDC1 genes. "Other genetic modifications" include any genetic modifications e.g. addition of plasmids, insertions, deletions or disruptions of one or more genes or partial genes and insertions, deletions or disruptions of one or more nucleotides. Also epigenetic modifications such as methylation are included in "other genetic modifications". Furthermore, the cell may
be genetically modified to produce or not to produce other compounds than styrene.

Also, it is an advantage of the present invention that strains can be constructed which overproduce phenylalanine, convert phenylalanine to cinnamic acid and subsequently to styrene. These strains provide the most efficient way for styrene production. In one embodiment of the invention the recombinant micro-organism or fungus further comprises the expression of a gene coding for a phenylalanine ammonia lyase, e.g. comprises the overexpression of the PAL gene.

The recombinant organism may also contain other genetic modifications than those specifically described herein. Methods for making any genetic modifications are generally well known and are described in various practical manuals describing laboratory molecular techniques. "Other genetic modifications" may be present in the cell prior to further modifying the cell according to the present invention for the overexpression of the PAD1 and FDC1, or may be done simultaneously with or after such further modifications.

One suitable method for accomplishing genetic modifications of micro-organisms is to clone the upstream and downstream flanking regions for the gene (which includes at least a portion of the coding region of the gene), produce a vector containing the cloned upstream and downstream flanks, and transform the micro-organism with the vector. The vector may also contain other genetic material such as a marker gene or a selection gene that is desirably inserted into the genome of the micro-organism. Genetic modification of the micro-organism is accomplished in one or more steps via the design and construction of appropriate vectors and transformation of the micro-organism cell with those vectors. Electroporation, protoplast-PEG and/or chemical (such as calcium chloride- or lithium acetate-based) transformation methods can be used. Vectors used in the present invention can be circularized or linearized and may contain restriction sites of various types for linearization or fragmentation. Useful vectors may for example be conveniently obtained from commercially available yeast or bacterial vectors.

Successful transformants can be selected using the attributes contributed by the marker or selection gene. Screening can be performed by PCR or Southern analysis to confirm that the desired genetic modifications (e.g. insertions) have taken place, to confirm copy number and to identify the point of
integration of genes into the micro-organism cell's genome. Activity and/or lack of activity of the enzymes can be confirmed using known assay methods.

**Micro-organisms**

The invention is not restricted to expression of PAD1 and FDC1 in *S. cerevisiae*. For example we also expressed *S. cerevisiae* PAD1 and FDC1 in *E. coli* and observed an increased cinnamic acid decarboxylase activity. In a preferred embodiment of the invention, the micro-organism is selected from the group consisting of bacteria, yeast, filamentous fungi, cyanobacteria, algae, and plant cells.

Bacteria are used in industry in a number of ways that generally exploit their natural metabolic capabilities. Suitable bacteria for production of styrene include but are not limited to *E. coli*.

Fungal species are in many cases the preferred industrial production organisms for example because of their low nutrition requirements and easy handling (rigid cell wall, big cells). In one embodiment of the invention a fungus has been genetically modified to overexpress the PAD1 and FDC1 genes. Fungus (adjective fungal) is used to refer to any organism belonging to the Kingdom *Fungi*, regardless of whether the organism grows primarily as yeast or as a filamentous organism. Suitable fungi include, for example, yeast cells of the genera *Saccharomyces*, *Kluveromyces*, *Candida*, *Pichia*, *Pachysolen* and *Hansenula*. Yeast species of particular interest include *S. cerevisiae*, *S. exigus*, *K. marxianus*, *K. lactis*, *K. thermotolerans*, *C. sonorense*, *C. krusei*, *C. shehatae*, *Pachysolen tannophilus* and *Pichia stipitis*. Suitable fungi also include, for example, filamentous fungi of the genera *Aspergillus*, *Trichoderma*, *Monascus*, and *Penicillium*. Fungal species of particular interest include *A. niger*, *A. ficuum*, *A. phoenicis*, *T. reesei*, *T. harzianum*, *M. ruber*, and *P. chrysogenum*. Preferred fungus include those of the species *S. cerevisiae*, *S. exigus*, *K. marxianus*, *K. lactis*, and *A. niger*. In one embodiment of the invention, the micro-organism or the fungus is selected from the group consisting of *Saccharomyces*, *Kluveromyces* and *Aspergillus*. Most preferably the fungus is *Saccharomyces cerevisiae*.

Producing high concentrations of styrene makes the method of the present invention economic. Indeed, in a preferred embodiment the micro-organism is resistant to styrene. Most preferably the micro-organism is tolerant to saturating concentrations of styrene in aqueous medium. Surprisingly growth of *S. cerevisiae* is not affected up to saturating concentrations of sty-
rene (Example 5). This means that there is virtually no toxic concentration of styrene for S. cerevisiae.

**Culturing recombinant micro-organisms and recovering styrene**

In the production process of the invention, a recombinant micro-organism is cultured in a growth and production medium that includes compounds for growth and energy. The medium used for producing styrene is any conventional medium, such as aqueous media, or solid media for culturing the micro-organisms of the invention. For example, suitable media include but are not limited to the following: Yeast Extract Peptone Dextrose (YPD) media, Luria Broth (LB) media or any medium suitable for culturing filamentous fungi, yeast or bacteria.

The carbon substrate used as a source for styrene production (Figure 1) may be provided as pure substrates or from complex sources. Carbon substrate can be any carbon substrate, which can be directly or through one or more steps converted to L-phenylalanine or to trans-cinnamic acid. In a preferred embodiment of the invention, the carbon substrate is glucose such as D-glucose or sugars that can be derived from hemicellulose such as pentoses. The medium may also contain alternative carbon sources, such as ethanol, glycerol, acetate, L-arabinose, D-galacturonate or amino acids.

In a method of converting trans-cinnamic acid to styrene "trans-cinnamic acid containing medium" refers to a medium that comprises cinnamic acid in a trans-stereoisomeric form (Figure 1).

In addition, the medium may consist of or contain complex, poorly defined elements, such as would be present in relatively inexpensive sources like corn steep liquor or solids, or molasses. Sugars of the fermentation medium are notably hexose sugars such as glucose, fructose, mannose, or galactose and oligomers of glucose such as maltose, maltotriose, isomaltotriose, starch or cellulose. In case of oligomeric sugars, it may be necessary to add enzymes to the fermentation broth in order to digest these to the corresponding monomeric sugar. The medium will typically contain nutrients required by the particular micro-organism, including a source of nitrogen (such as amino acids, proteins, inorganic nitrogen sources such as ammonia or ammonium salts), and various vitamins and minerals. Also any other agents or compounds may be present in the medium according to the common general knowledge of the art. Thus, the trans-cinnamic acid or carbon substrate containing medium
may further comprise any of the conventional or above mentioned agents.

Other fermentation conditions, such as temperature, cell density, selection of nutrients, and the like are not considered to be critical to the invention and are generally selected to provide an economical process. Temperatures during each of the growth phase and the production phase may range from above the freezing temperature of the medium to about 50°C, although the optimal temperature will depend somewhat on the particular microorganism. A preferred temperature, particularly during the production phase, is from about 25 to 30°C, most preferably about 30°C.

The pH of the process may or may not be controlled to remain at a constant pH, but should preferably be between about 3.0 and 6.5, depending on the production organism. Optimally the pH is controlled to a constant pH of 3.5 to 5.5. Suitable buffering agents include, for example, calcium hydroxide, calcium carbonate, sodium hydroxide, potassium hydroxide, potassium carbonate, sodium carbonate, ammonium carbonate, ammonia, ammonium hydroxide and the like. In general, those buffering agents that have been used in conventional fermentation processes are also suitable here. It is within the scope of the invention, however, to allow the pH of the fermentation medium to drop from a starting pH that is typically 6 or higher, to below the pKa of the acid fermentation product, such as in the range of about 3 to about 4.

The fermentation is conducted aerobically or microaerobically. If desired, a specific oxygen uptake rate can be used as a process control. The process of the invention can be conducted continuously, batch-wise, or any combination thereof.

After culturing the recombinant micro-organisms of the invention the resulting styrene is recovered from the medium. Effects of styrene concentrations on the growth of micro-organisms were tested in Example 5. S. cerevisiae was grown in liquid (aqueous) medium with increasing amounts of styrene. Up to 5 g/l styrene was added and no change in the growth rate was observed. Thus, styrene even in high concentrations is not toxic for S. cerevisiae. 5 g/l is by far exceeding the solubility of styrene in water. At 30 °C, where the growth was measured, the solubility is about 0.36 g/l. If more styrene is produced a phase separation occurs and styrene will float on the fermentation broth (styrene has with 0.91 g/l a lower density than water). Therefore, lots of styrene can be produced and furthermore, it will be easily collected.
In one embodiment of the invention the method of producing styrene or converting trans-cinnamic acid to styrene further comprises an optional step d) of isolating and purifying the styrene from the medium. Styrene may be isolated and purified from the medium by using any conventional methods known in the art such as ion exchange, two phase extraction or distillation.

It will be obvious to a person skilled in the art that, as the technology advances, the inventive concept can be implemented in various ways. The invention and its embodiments are not limited to the examples described above but may vary within the scope of the claims.

EXAMPLES

**Example 1: Expression of the S. cerevisiae genes PAD1 and FDC1 in S. cerevisiae**

The FDC1 and PAD1 genes were cloned by PCR from genomic DNA of the *S. cerevisiae* strain S288C.

The FDC1 was amplified with the primers 8796: GAGGAATTCAAAATGAGGAAGCTAAATCCAGCT (SEQ ID NO:1) and 8798: GAGGCTAGCCCGTAGAAAGTCTATGGCAATTA (SEQ ID NO:2). With these primers EcoRI and Nhel restriction sites were introduced. The PCR product was digested with EcoRI and Nhel and ligated to the corresponding sites of the plasmid p2158. This plasmid was derived from the pYX242 by changing the multiple cloning sites. The resulting plasmid is a yeast expression vector expressing the FDC1 under the TPII promoter and with LEU2 for selection.

The PAD1 was amplified with the primers 8803 AAAGAATTCTTACTTGGCTTTTTATCTCCTTCCAACG (SEQ ID NO:3) and 8804: ATACTCGAGAAATGCTCCTATTTCAAGAGAAGACT (SEQ ID NO:4) to introduce the restriction sites Xhol and EcoRI. The PCR product was digested with Xhol and EcoRI and ligated to the pFL60. The resulting plasmid is a yeast expression vector expressing the PAD1 under PGK1 promoter and URA3 selection.

Both plasmids were then transformed to the *S. cerevisiae* strain CEN.PK2-1 C and grown on a selective medium lacking leucine and uracil. The resulting strain was then tested for growth on plates in the presence of cinnamic acid. The concentrations 100 µM, 500 µM, 1 mM, 3 mM,
5 mM, 10 mM and 20 mM cinnamic acid were tested. The strain expressing PAD1 and FDC1 was growing up to concentrations of 10 mM cinnamic acid. A control strain, containing the plasmids without FDC1 and PAD1 showed reduced growth at 500 μM and 1 mM cinnamic acid but no growth at higher concentrations.

The cell extract was assessed for cinnamic acid decarboxylase activity. For that a cell extract was made using glass beads and a bead beater. The cell extract was centrifuged and the supernatant analysed for decarboxylase activity. Cinnamic acid at a concentration of about 0.5 mM in 20 mM sodium phosphate buffer pH 7.0 and cell extract with a final protein concentration of 0.1 mg/ml were incubated at different time intervals and the reaction was stopped by keeping the solution at 96°C for 10 minutes. The disappearance of cinnamic acid was followed by UPLC using an Acquity UPLC BEH C18 1.7μm column (2.1 x 100 mm) with a Waters Acquity UPLC system. The results of the decarboxylase activity of strains with the S. cerevisiae PAD1 and/or FDC1 are shown in Figure 2. Figure 2 reveals that only upon the expression of the both FDC1 and PAD1 significant cinnamic acid decarboxylation is observed.

Example 2: Expression of an FDC1 homologue from Aspergillus niger in S. cerevisiae

To identify a FDC1 homologue in Aspergillus niger a BLAST search was performed to identify the gene with the highest homology. The gene coding for the FDC1 homologue in the Aspergillus niger strain ATCC1015 has an intron. In order to express only the open reading frame the two exons were amplified by PCR and assembled with a yeast expression vector by homologues recombination in yeast. The exons were amplified by PCR by using the primer pairs: primer 8850 TGCTTAAATCTATAACTACAAAAACATA-CAGGAATTCCATGTCTCGCAACCTGCTC (SEQ ID NO:5) / primer 8851 CCAGAGAGCCGATCATGCTGCTGCTTTCAATGGATCAACCGGC (SEQ ID NO:6) and primer 8852 GCCGCTTGAGCGATGAAACGCACAC-CATGATCAGCTGACTCTCTG (SEQ ID NO:7) / primer 8853 CAGGCTAGCTACTGACTCGACTCTAGAGGATCTTTATTTTTT-GCTGAAACCCATCAAC (SEQ ID NO:8) and genomic DNA of the A. niger strain ATCC1015. The PCR products and the EcoRI/BamHI digested plasmid p2158 were used to transform the yeast strain CEN.PK2-1 C. Transformants
were screened for growth in the absence of leucine. From positive transformants the plasmid was rescued and sequenced.

The plasmid with the A. niger FDC1 homologue and the plasmid with the S. cerevisiae PAD1 were then transformed to the S. cerevisiae strain CEN.PK2-1 C and grown on selective medium lacking leucine and uracil.

The resulting strain was then tested for growth on plates in the presence of cinnamic acid. The concentrations 100 µM, 500 µM, 1 mM, 3 mM, 5 mM, 10 mM and 20 mM cinnamic acid were tested. The resulting strain with the A. niger FDC1 and S. cerevisiae PAD1 showed a similar behavior as the strain with the FDC1 and PAD1 form S. cerevisiae overexpressed as described in example 1.

Example 3: Expression of the S. cerevisiae PAD1 and FDC1 in E. coli

The PAD1 and FDC1 from S. cerevisiae were expressed with the aid of the pACYCDuet-1 expression vector (Novagen). The FDC1 was amplified by PCR from genomic DNA of the S. cerevisiae strain S288C introducing an Ncol and a HindIII restriction site. The primers were 8846: GTAGTAC-CATGGGGGATGAGGAAGCTAAATCCAGCTT (SEQ ID NO:9) and 8847: GTAGTAAGCTTTTATTTATACCTTTTCCAA (SEQ ID NO:10). The PCR product was then digested with Ncol and HindIII and ligated to the corresponding sites of the pACYCDuet-1.

The PAD1 was amplified by PCR from the same template and the restriction sites Ndel and Xhol introduced. The primers were 8848: GTAGTCATATGCTCCTATTTCAAGAAGAACT (SEQ ID NO:11) and 8849: GTAGCTCGAGTTACTTGTCTTTTTATTCCTTTCCA (SEQ ID NO:12). The PCR product was then digested with Ndel and Xhol and the ligated to the corresponding sites of the pACYCDuet-1 containing the FDC1.

The resulting plasmid was then transformed to the E. coli strain BL21 (DH3) and selected for chloramphenicol resistance. The strain was grown on LB at 37 °C with chloramphenicol to an optical density of 0.4 at 600 nm and the protein production induced with IPTG for 3 hours. The cells were harvested by centrifugation and lysed in a lysis buffer containing 0.1 mg/ml lysozyme, 0.1 % Triton-X1 00 0.5 mM EDTA, 0.5 M NaCl and 20 mM Tris-Cl pH 8.0 for 30 minutes on ice and ultrasonication. The extract was centrifuged in a Sorval centrifuge with an SS-34 rotor at 20,000 rpm and the supernatant analysed for
frans-cinnamic acid decarboxylase activity as in example 1. The results are shown in Figure 3.

As seen from Figure 3 expression of the FDC1 and PAD1 gives a higher rate of cinnamic acid decarboxylation as compared to expressing FDC1 or PAD1 alone.

Example 4: Expression of a PAD1 homologue from Aspergillus niger in S. cerevisiae

To express the Aspergillus niger PAD1 homologue in S. cerevisiae the mitochondrial targeting sequence was modified. The mitochondrial targeting sequence of S. cerevisiae MLLFPRRTNIAFFKTGIFANFPLLGRIT TSPSFLTHKLSKEVTRASTSPPRPKRI (SEQ ID NO:13) was used to replace the sequence MFNSLLSGTTTPNSGRASPPASEMPIDNDHVAVARPAPRRRI (SEQ ID NO:14) of the A. niger protein. The gene was custom synthetized and expressed from the pFL60 plasmid as in the Example 1. A yeast strain expressing the A. niger PAD1 homologue and the S. cerevisiae FDC1 was constructed using the FDC1 expression vector as in the Example 1. The resulting strain showed increased resistance toward cinnamic acid as in the Example 1.

Example 5: Testing the toxicity of styrene for S. cerevisiae

In order to evaluate the suitability of the yeast S. cerevisiae for styrene production we tested at what concentrations styrene is toxic for this yeast. For that purpose an S. cerevisiae strain was grown aerobically in rich glucose medium that was supplied with increasing concentrations of styrene at a temperature of 30°C and the growth rates were measured. The growth rates were identical up to concentrations of 5 g/l of styrene. 5 g/l styrene is already exceeding the solubility of styrene in water under these conditions.

Example 6: Expression of PAD1, FDC1 and PAL in S. cerevisiae

We constructed an S. cerevisiae yeast strain in which the following genes were overexpressed: (i) the PAL gene from Rhodotorula rubra (coding for phenylalanine ammonia lyase, Vaslet et al., GenBank accession code: X13094.1), as single open reading frame and codon optimized for expression in yeast, (ii) the FDC1 from S. cerevisiae and (iii) the PAD1 from S. cerevisiae. For the PAL gene a cassette was constructed where the PAL was under the S. cerevisiae TPI1 promoter. The cassette also contained the kanMX marker be-
tween ΙοχΡ sites and ARO10 flanking sites for targeted integration to replace the ARO10 gene. The S. cerevisiae strain CEN.PK1 10-1 0C was transformed with this cassette and KanMX marker looped out. The Pad1 gene was amplified by PCR to introduce EcoRI and HindIII restriction sites and ligated to the pYX022 integration vector. This expression vector has the S. cerevisiae TPI1 promoter and the S. cerevisiae HIS3 for targeted integration. The strain harbouring the PAL gene from above was transformed with the Ndel linearized vector. For the FDC1 the gene including promoter and terminator from Example 1 was amplified by PCR to introduce Sail and BgIII restriction sites and ligated to the corresponding sites of the plasmid M4297 (ATCC 87804). This plasmid has the KanMX gene for selection and HO flanking sites for targeted integration to the HO locus. The integration cassette was released by NotI digestion and was used to transform the strain overexpressing PAL and PAD1 from above. The resulting strain was overexpressing PAD, PAD1 and FDC1 and was free of auxotrophies.

The strain showed cinnamic acid resistance similar to the strain from example 1 and phenylalanine ammonia lyase and cinnamic acid decarboxylase activity was confirmed from the cell extract. The resulting strain was also used to test the resistance toward 4-fluoro-phenylalanine. Screening for 4-fluoro-phenylalanine resistance is widely used to screen for phenylalanine overproducing mutants and has been used in S. cerevisiae as described by Fukuda et al (1991). The result is shown in Figure 4A (and the control strain in Figure 4B). Growth is shown in the presence of different concentrations of 4-fluoro-phenylalanine. Figure 4A reveals that the strain is still sensitive to 4-fluoro-phenylalanine, i.e. 4-fluoro-phenylalanine can still be used for screening. However the sensitivity is decreased meaning that part of the 4-fluoro-phenylalanine is converted by the enzymes Pal, Fdc1 and Pad1.

References


Claims

1. A method of producing styrene, said method comprising
   a) providing a recombinant micro-organism that has been genetical-
      ly modified to overexpress the PAD1 and FDC1 genes,
   b) culturing said recombinant micro-organism in a carbon substrate
      containing medium to obtain styrene, and
   c) recovering the resulting styrene from the medium.

2. A method of converting \textit{trans}-cinnamic acid to styrene, said
   method comprising
   a) providing a recombinant micro-organism that has been genetical-
      ly modified to overexpress the PAD1 and FDC1 genes,
   b) culturing said recombinant micro-organism in a \textit{trans}-cinnamic
      acid containing medium to obtain styrene, and
   c) recovering the resulting styrene from the medium.

3. The method of claim 1 or 2, wherein the method further compris-
   es an optional step d) of isolating and purifying the styrene from the medium.

4. Use of a recombinant micro-organism that has been genetically
   modified to overexpress the PAD1 and FDC1 genes for producing styrene of a
   carbon substrate or for converting \textit{trans}-cinnamic acid to styrene.

5. The method of any one of claims 1-3 or the use of claim 4, where-
   in the carbon substrate is glucose or sugars derived from hemicellulose.

6. The method or use of any one of claims 1-5, wherein the micro-
   organism is selected from the group consisting of bacteria, yeast, filamentous
   fungi, cyanobacteria, algae, and plant cells.

7. The method or use of any one of claims 1-6, wherein the micro-
   organism is a fungus selected from the group consisting of \textit{Saccharomyces},
   \textit{Kluyveromyces} and \textit{Aspergillus}.

8. The method or use of any one of claims 1-7, wherein the micro-
   organism is resistant to styrene.

9. A recombinant fungus that has been genetically modified to over-
   express the PAD1 and FDC1 genes.

10. The recombinant fungus of claim 9, wherein the fungus is se-
    lected from the group consisting of \textit{Saccharomyces}, \textit{Kluyveromyces} and \textit{As-
        pergillus}, preferably the fungus is \textit{Saccharomyces cerevisiae}. 
11. The methods, uses or recombinant fungi of any one of claims 1-10, wherein at least one of the PAD1 and FDC1 genes is derived from a yeast, a filamentous fungus, or a bacterium.

12. The methods, uses or recombinant fungi of any one of claims 1-11, wherein at least one of the PAD1 and FDC1 genes is derived from Saccharomyces cerevisiae, E.coli, or Aspergillus niger.

13. The methods, uses or recombinant fungi of any one of claims 1-12, wherein the recombinant micro-organism or fungus further comprises other genetic modifications.

14. The methods, uses or recombinant fungi of any one of claims 1-13, wherein the recombinant micro-organism or fungus further comprises the expression of a gene coding for a phenylalanine ammonia lyase.
Figure 1.
Figure 2.
Figure 3.
Growth curves of strain expressing PAL, PAD1 and FDC1 in the presence 4-fluoro-phenylalanine.

- 1 g/L
- 0.5 g/L
- 0.25 g/L
- 0.06 g/L
- 0.03 g/L

Figure 4A.
Figure 4B. Growth curves of control strain in the presence of 4-fluorophenylalanine.
**INTERNATIONAL SEARCH REPORT**

International application No
PCT/FI2014/05Q498

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12P5/00 C12N1/15
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, FSTA, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C.

See patent family annex.

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

Authorized officer
Korsner, Sven-Erik
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