(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 October 2010 (21.10.2010)

PCT

(10) International Publication Number
WO 2010/118932 A1

(51) International Patent Classification:
C12P 7/46 (2006.01)

(21) International Application Number:
PCT/EP2010/053753

(22) International Filing Date:
23 March 2010 (23.03.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

09157964.9	15 April 2009 (15.04.2009)	EP
09169412.5	3 September 2009 (03.09.2009)	EP
61/239,566	3 September 2009 (03.09.2009)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/118932 A1

(54) Title: DICARBOXYLIC ACID PRODUCTION PROCESS

(57) Abstract: The present invention relates to a process for the production of a dicarboxylic acid and ethanol which comprises fermenting a genetically modified yeast in a suitable fermentation medium under anaerobic conditions at a pH value of between 1 and 5 and producing the dicarboxylic acid and ethanol. The invention also relates to a process for crystallizing succinic acid from an aqueous solution having a pH of between 1 and 5 and comprising succinic acid, comprising removing part of the aqueous solution by evaporation to obtain a concentrated solution, and bringing the temperature of the concentrated solution to a value of between 10 and 30 degrees Celsius, wherein succinic acid crystals are formed.

DICARBOXYLIC ACID PRODUCTION PROCESS

The present invention relates to a process for the fermentative production of a dicarboxylic acid by yeast and the recovery of the dicarboxylic acid by crystallization.

Dicarboxylic acids such as malic acid, fumaric acid and succinic acid, are important compounds which are used in the food industry for the preparation and preservation of food, in the medical industry for the formulation of medical products, building blocks in (bio)polymers and other industrial uses. To meet the increasing need for dicarboxylic acids, more efficient and cost effective production methods are being developed.

Bacteria can produce large amounts of dicarboxylic acids. However, one major drawback associated with the use of bacteria for producing dicarboxylic acids is the formation of the acid salt. If bacteria are used, the pH needs to be maintained in the range of pH 6-7. As a consequence, most acids will be produced in their salt form and the salts will have to be converted into the acid. This is not practical or efficient in large-scale production processes and raises production costs. Alternatively, low pH processes are considered to be more attractive, in particular at a large scale.

Yeasts are attractive candidates for low pH dicarboxylic acid production processes. However, at low pH a dicarboxylic acid in the acid form diffuses through the cell membrane inside the cell, whereupon the cell will try to transport the dicarboxylic acid outside the cell. This transport will likely require energy in the form of ATP. Under aerobic conditions, a yeast cell can generate energy in the form of ATP by oxidative respiration. An aerobic process for the production of malic acid and succinic acid by a (genetically modified) yeast is for instance disclosed in WO2007/061590. However, aerobic processes for the production of dicarboxylic acid by yeast fermentation are not desirable since under aerobic conditions the dicarboxylic acid yield on carbon is estimated to be lower than under anaerobic conditions.

Under anaerobic conditions, yeast produces dicarboxylic acid via the reductive tricarboxylic acid cycle, producing 1 mole of ATP, which is considered not to be sufficient for maintenance of the cell and/or transport of the dicarboxylic acid outside the cell. Therefore, the yeast cell will need to use alternative ways to

generate energy during anaerobic dicarboxylic acid fermentation, with little to no expense of the dicarboxylic acid yield.

The aim of the present invention is a process for the production of a dicarboxylic acid by yeast fermentation under anaerobic conditions and at low pH, wherein the dicarboxylic acid is produced at a high amount.

The aim is achieved according to the invention with a process for the production of a dicarboxylic acid and ethanol which comprises fermenting a genetically modified yeast in a suitable fermentation medium under anaerobic conditions at a pH value of between 1 and 5 and producing the dicarboxylic acid and ethanol.

Surprisingly, it was found that ethanol production by the genetically modified yeast in the process according to the present invention produced sufficient ATP for maintenance of the yeast cell and at the same time allowed dicarboxylic acid production at a high amount under anaerobic conditions. The stoichiometric reaction of ethanol from glucose is : 1 glucose $\rightarrow$ 2 ethanol + 2 CO_2 + 2 ATP.

WO89/05861 discloses a yeast fermentation process for the production of ethanol and succinic acid. However, the fermentation process disclosed in WO89/05861 resulted in low amounts of succinic acid and high amounts of ethanol.

A high amount of dicarboxylic acid as used herein is defined as an amount of dicarboxylic acid of at least 1 g/l under anaerobic conditions and a pH of between 1 and 5, preferably an amount of at least 2, 5, 10 g/l, preferably at least 12, 15 g/l, more preferably at least 18 g/l, usually below 100 g/l of dicarboxylic acid in the process for the production of dicarboxylic acid and ethanol according to the present invention.

The dicarboxylic acid in the process of the invention preferably is malic acid, fumaric acid or succinic acid, most preferably succinic acid. The term dicarboxylic acid is used to indicate also dicarboxylate, such as malate, fumarate and succinate which are used interchangeably. Dicarboxylate is the ionic form of dicarboxylic acid.

In another embodiment, ethanol in the process according to the present invention is produced in a low amount. A low amount of ethanol as used herein is an amount of below 100 g/l, preferably below 80, 60, 40 g/l, preferably below 35 g/l, preferably below 30 g/l, and usually more than 1 g/l.

It was found advantageous that a lower amount of ethanol was produced by the genetically modified yeast as compared to a wild type yeast since which resulted in the production of a higher amount of dicarboxylic acid.

Another advantage for the production of ethanol in the process for the production of dicarboxylic acid according to the present invention is that simultaneously carbondioxide (CO_2) is produced. A higher amount of CO_2 leads to a higher production of oxaloacetate from pyruvate, eg. by phosphoenol pyruvate carboxykinase, which positively influences the production of a dicarboxylic acid in the process according to the present invention.

Another advantage of the process according to the invention was that the process was suitable to be implemented in existing ethanol fermentation facilities.

Surprisingly, it was found that in the process according to the present invention low amounts of by-products such as organic acids were formed. In particular, it was surprisingly found that a low amount of glycerol was formed. A low amount of glycerol is herein defined as a concentration of below 2 g/l, preferably below 1 g/l, preferably below 0.5 g/l, usually above 0.01 g/l.

The process for the production of dicarboxylic acid and ethanol according to the present invention may be carried out in any suitable mode, such as a batch, fed-batch, continuous mode or a combination of these fermentation modes. Preferably, the process for the production of dicarboxylic acid and ethanol according to the present invention is carried out in a fed-batch mode or continuous mode.

In one embodiment fermenting the yeast in the process of the invention is carried out under carbohydrate limiting conditions. As used herein, carbohydrate limiting conditions are defined as maintaining the carbohydrate concentration below 10 g/l, preferably below 5 g/l preferably below 2 g/l, usually above 0.5 g/l.

The process for the production of dicarboxylic acid and ethanol according to the present invention may be carried out in any suitable volume and scale, preferably on an industrial scale. Industrial scale is defined herein as a volume of at least 10, or 100 litres, preferably at least 1 cubic metre, preferably at least 10, or 100 cubic metres, preferably at least 1000 cubic metres, usually below 10,000 cubic metres.

Fermenting the genetically modified yeast in the process of the invention may be carried out in any suitable fermentation medium comprising a suitable nitrogen source, carbohydrate and other nutrients required for growth and production of a dicarboxylic acid and ethanol in the process of the invention. A suitable carbohydrate in the fermentation process according to the invention may be glucose, galactose, xylose, arabinose, sucrose, or maltose.

An anaerobic fermentation process is herein defined as a fermentation process run in the absence of oxygen, and wherein organic molecules serve as both electron donor and electron acceptor.

In one embodiment, the fermentation process is carried out under a partial CO_2 pressure of between 5% and 50%, preferably between 10% and 40%.

The pH during the process for the production of dicarboxylic acid and ethanol usually lowers during the production of the dicarboxylic acid. Preferably, the pH in the process for the production of dicarboxylic acid and ethanol ranges between 1 and 5, preferably between 1.5 and 4.5, more preferably between 2 and 4.

In another preferred embodiment the process according to the present invention comprises a step of preculturing the genetically modified yeast under aerobic conditions in the presence of a carbohydrate. . Preferably, the fermenting of the yeast is carried out at a pH of between 4 and 6. Preferably, the carbohydrate during preculturing is a non-repressing carbohydrate, preferably galactose. It was found advantageous to preculture yeast on a non-repressing carbohydrate, since this prevented glucose repression to occur in yeast, which negatively influences the amount of dicarboxylic acid produced. In addition, it was found that a step of preculturing the yeast under aerobic conditions resulted in a higher biomass yield and a faster growth. Preferably, the preculturing is carried out in batch mode.

A genetically modified yeast in the process of the invention, may comprise any suitable genetic modifications, such as deletions or disruptions, and insertions of homologous or heterolgous nucleotides sequences. A yeast in the process of the invention may be genetically modified or transformed with nucleotide sequences that encode homologous and/or heterologous enzymes that catalyse reactions in the cell resulting in an increased flux towards a dicarboxylic acid such malic acid, fumaric acid and/ or succinic acid. It may for example be favourable to introduce and/or overexpress nucleotide sequences encoding i) a malate dehydrogenase which catalyses the conversion from OAA to malic acid; ii) a fumarase, which catalyses the conversion of malic acid to fumaric acid; or iii) a fumarate reductase that catalyses the conversion of fumaric acid to succinic acid, depending on the dicarboxylic acid to be produced.

It was found that a genetically modification of a yeast cell in a process according to the present invention was essential to obtain a high amount of dicarboxylic acid and a low amount of ethanol. Preferably, a yeast cell in the process according to the present

invention comprises genetic modifications according to the preferred embodiments as described herein below.

Preferably the genetically modified yeast expresses a nucleotide sequence encoding a phosphoenolpyruvate (PEP) carboxykinase in the cytosol. Preferably a nucleotide sequence encoding a phosphoenolpyruvate (PEP) carboxykinase is overexpressed. The PEP carboxykinase (EC 4.1.1.49) preferably is a heterologous enzyme, preferably derived from bacteria, more preferably the enzyme having PEP carboxykinase activity is derived from *Escherichia coli, Mannheimia sp., Actinobacillus sp.,* or *Anaerobiospirillum sp.,* more preferably *Mannheimia succiniciproducens,* Preferably, a yeast cell according to the present invention is genetically modified with a PEP carboxykinase which has at least 80, 85, 90, 95, 99 or 100% sequence identity with amino acid sequence of SEQ ID NO: 6.

In another preferred embodiment a genetically modified yeast in the process according to the present invention further expresses a nucleotide sequence encoding a malate dehydrogenase (MDH; E.C. 1.1.1.37) which is active in the cytosol upon expression of the nucleotide sequence. Preferably the malate dehydrogenase is overexpressed. A cytosolic MDH may be any suitable homologous or heterologous malate dehydrogenase. Preferably, the MDH is *S. cerevisiae* MDH2 which has been modified such that it is not inactivated in the presence of glucose and is active in the cytosol. It is known that the transcription of *MDH2* is repressed and Mdh2p is degraded upon addition of glucose to glucose-starved cells. Mdh2p deleted for the first 12 amino-terminal amino acids is less-susceptible for glucose-induced degradation (Minard and McAlister-Henn, J. Biol Chem. 1992 Aug 25;267(24):17458-64). Preferably, a yeast cell according to the present invention comprises a nucleotide sequence encoding a malate dehydrogenase that has at least 70%, preferably at least 75, 80, 85, 90, 92, 94, 95, 96, 97, 98, 99% or 100% sequence identity with the amino acid sequence of SEQ ID NO: 9.

In another preferred embodiment a genetically modified yeast in the process according to the present invention expresses a nucleotide sequence encoding a fumarase (E.C. 4.2.1.2.) in the cytosol, which may be a heterologous or homologous enzyme. Preferably a nucleotide sequence encoding a fumarase is overexpressed. A nucleotide sequence encoding an heterologous fumarase may be derived from any suitable origin, preferably from microbial origin, preferably from a yeast, for instance *Saccharomyces cerevisiae* or a filamentous fungus, for instance *Rhizopus oryzae*.

Preferably, a yeast in the process according to the present invention overexpresses a nucleotide sequence encoding a fumarase that has at least 70%, preferably at least 75, 80, 85, 90, 92, 94, 95, 96, 97, 98, or 99% or 100% sequence identity with the amino acid sequence of SEQ ID NO: 8.

In another preferred embodiment a genetically modified yeast in the process according to the present invention expresses a nucleotide sequence encoding a fumarate reductase (E.C. 1.3.1.6) in the cytosol. Preferably, the fumarate reductase is a heterologous enzyme, preferably a NAD(H)-dependent fumarate reductase, which may be derived from any suitable origin, for instance bacteria, fungi, protozoa or plants. Preferably, the (NAD(H)-dependent) fumarate reductase is overexpressed. Preferably, a yeast in the process according to the invention comprises a heterologous NAD(H)-dependent fumarate reductase, preferably derived from a *Trypanosoma* sp, for instance a *Trypanosoma brucei.* Preferably, a yeast cell according to the present invention is genetically modified with a NAD(H)-dependent fumarate reductase,which has at least 80, 85, 90, 95, 99 or 100% sequence identity with SEQ ID NO: 7.

In another embodiment, a genetically modified yeast in the process according to the invention expresses a nucleotide sequence encoding a dicarboxylic acid transporter protein, preferably a malic acid transporter protein (MAE) in the cytosol. Preferably the dicarboxylic acid transporter protein is overexpressed. A dicarboxylic acid transporter protein may be any suitable homologous or heterologous protein. Preferably the dicarboxylic acid transporter protein is a heterologous protein. A dicarboxylic acid transporter protein may be derived from any suitable organism, preferably from yeast or fungi such as *Schizosaccharomyces pombe*. Preferably, a dicarboxylic acid transporter protein is a malic acid transporter protein (MAE) which has at least 80, 85, 90, 95 or 99% or 100% sequence identity with SEQ ID NO: 10.

Preferably, the yeast in the process of the invention comprises at least one gene encoding glycerol-3-phosphate dehydrogenase which is not functional. A glycerol-3-phosphate dehydrogenase gene that is not functional is used herein to describe a eukaryotic cell, which comprises a reduced glycerol-3-phosphate dehydrogenase activity, for instance by mutation, disruption, or deletion of the gene encoding glycerol-3-phosphate dehydrogenase, resulting in a decreased formation of glycerol as compared to a wild-type cell.

Preferably, a genetically modified yeast in the process according to the present invention overexpresses a nucleotide sequence encoding a PEP carboxykinase, a

nucleotide sequence encoding a malate dehydrogenase, a nucleotide sequence encoding a fumarase, a nucleotide sequence encoding a NAD(H) dependent fumarate reductase, and/or a nucleotide sequence encoding a malic acid transporter protein, preferably wherein the enzymes are active in the cytosol. Preferred embodiments of the enzymes are as described herein above.

As used herein, a genetically modified yeast according to the present invention is defined as a cell which contains, or is transformed or genetically modified with or a nucleotide sequence or polypeptide that does not naturally occur in the yeast cell, or it contains additional copy or copies of an endogenous nucleic acid sequence, or it contains a deletion or disruption of an endogenous or homolgous nucleotide sequence. A wild-type eukaryotic cell is herein defined as the parental cell of the recombinant cell.

The term "homologous" when used to indicate the relation between a given (recombinant) nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean that in nature the nucleic acid or polypeptide molecule is produced by a host cell or organism of the same species, preferably of the same variety or strain.

The term "heterologous" when used with respect to a nucleic acid (DNA or RNA) or protein refers to a nucleic acid or protein that does not occur naturally as part of the organism, cell, genome or DNA or RNA sequence in which it is present, or that is found in a cell or location or locations in the genome or DNA or RNA sequence that differ from that in which it is found in nature. Heterologous nucleic acids or proteins are not endogenous to the cell into which it is introduced, but have been obtained from another cell or synthetically or recombinantly produced.

Sequence identity is herein defined as a relationship between two or more amino acid (polypeptide or protein) sequences or two or more nucleic acid (polynucleotide) sequences, as determined by comparing the sequences. Usually, sequence identities or similarities are compared over the whole length of the sequences compared. In the art, "identity" also means the degree of sequence relatedness between amino acid or nucleic acid sequences, as the case may be, as determined by the match between strings of such sequences.

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include BLASTP and BLASTN,

publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894). Preferred parameters for amino acid sequences comparison using BLASTP are gap open 11.0, gap extend 1, Blosum 62 matrix.

The term "nucleic acid" as used herein, includes reference to a deoxyribonucleotide or ribonucleotide polymer, i.e. a polynucleotide, in either single-or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids). A polynucleotide can be full-length or a subsequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence thereof.

There are various means available in the art for overexpression of nucleotide sequences encoding enzymes in a yeast in the process of the invention. In particular, a nucleotide sequence encoding an enzyme may be overexpressed by increasing the copy number of the gene coding for the enzyme in the cell, e.g. by integrating additional copies of the gene in the cell's genome, by expressing the gene from a centromeric vector, from an episomal multicopy expression vector or by introducing an (episomal) expression vector that comprises multiple copies of the gene. Preferably, overexpression of the enzyme according to the invention is achieved with a (strong) constitutive promoter.

The yeast in the process for the production of a dicarboxylic acid and ethanol according to the present invention preferably belongs to one of the genera *Schizosaccharomyces*, *Saccharomyces*, *Yarrowia*, *Candida*, *Pichia, Kluyveromyces, Issatchenko* or *Zygosaccharomyces.* More preferably, the yeast is a *Saccharomyces cerevisiae, Saccharomyces uvarum, Saccharomyces bayanus, Yarrowia lipolytica, Candida sonorensis, Schizosaccharomyces pombe, Pichia stipidis, Kluyveromyces marxianus, K. lactis, K. thermotolerans, Issatchenko orientalis* or *Zygosaccharomyces bailii.*

In another aspect the present invention relates to a process for producing a fermentation product by a genetically modified yeast, wherein ethanol production in the yeast is used to generate energy in the form of ATP under anaerobic conditions and a pH of between 1 and 5 in a suitable fermentation medium, and wherein the yeast comprises a genetic modification that increases the production of the fermentation

product as compared to a yeast not comprising said genetic modification. Surprisingly it was found that under low pH and anaerobic conditions production of ethanol, preferably a low amount of ethanol resulted in a high amount of fermentation product.

Preferably, the fermentation product is a dicarboxylic acid, preferably malic acid, fumaric acid or succinic acid. Preferred embodiments of the process for producing a fermentation product are further as defined herein for the process for the production of dicarboxylic acid and ethanol.

In a preferred embodiment the process for the production of a dicarboxylic acid and ethanol further comprises recovering the dicarboxylic acid and / or ethanol. Recovery of the dicarboxylic acid and ethanol may be carried out by any suitable method. Preferably, ethanol is recovered by distillation from the fermentation medium.

In a preferred embodiment the recovering of dicarboxylic acid comprises crystallizing the dicarboxylic acid and forming dicarboxylic acid crystals. Preferably, the crystallizing of dicarboxylic acid comprises removing part of the fermentation medium, preferably by evaporation, to obtain a concentrated medium.

In a preferred embodiment the process according to the present invention comprises recovering a dicarboxylic acid which is a succinic acid and wherein the recovering comprises crystallizing succinic acid from an aqueous solution having a pH of between 1 and 5 and comprising succinic acid, comprising evaporating part of the aqueous solution to obtain a concentrated solution, lowering the temperature of the concentrated solution to a value of between 5 and 35 degrees Celsius, wherein succinic acid cystals are formed. Preferably, the crystallizing comprises bringing the temperature of the concentrated medium to a temperature of between 10 and 30 degrees Celsius, preferably between 15 and 25 degrees Celsius. Preferably, the fermentation medium has a pH of between 1.5 and 4.5, preferably between 2 and 4.

It was found that crystallizing succinic acid at higher temperatures such as between 10 and 30 degrees Celsius resulted in succinic acid crystals with a lower amount of impurities such as organic acid, protein, color and/or odor, than succinic acid crystals that were crystallized at a low temperature of below 10 degrees.

Another advantage of crystallizing succinic acid at a higher temperature was that it requires a lower amount of energy for cooling the aqueous solution as compared to a process wherein crystallizing succinic acid is carried out below 10 or 5 degrees Celsius, resulting in a more economical and sustainable process.

Preferably, the crystallizing of succinic acid comprises a step of washing the succinic acid crystals.

Surprisingly it was found that it was possible to crystallize succinic acid directly from the fermentation medium having a pH of between 1 and 5 to a purity of at least 90% w/w, preferably at least 95, 96, 97, or at least 98%, or 99 to 100%w/w.

Preferably, the recovering of the dicarboxylic acid and ethanol, preferably succinic acid and ethanol, comprises removing the biomass from the fermentation medium, distilling ethanol from the fermentation medium and crystallizing the dicarboxylic acid, preferably crystallizing as described herein above. Preferably, the removing of biomass is carried out by filtration.

In a preferred embodiment the process for the production of a dicarboxylic acid and ethanol further comprises using the dicarboxylic acid and ethanol in an industrial process. An industrial process for a dicarboxylic acid may be the application as a cosmetic additive, deicing agent, food additive or as a building block for (bio)polymers. Industrial processes for ethanol may be the application as a solvent or as a biofuel.

In a preferred embodiment the fermentation medium comprises an amount of succinic acid of between 1 and 150 g/l, preferably between 5 and 100 g/l, more preferably between 10 and 80 g/l or between 15 and 60 g/l of succinic acid.

In another aspect the present invention relates to a process for crystallizing succinic acid from an aqueous solution having a pH of between 1 and 5 and comprising succinic acid, comprising removing part of the aqueous solution by evaporation to obtain a concentrated solution, and bringing the temperature of the concentrated solution to a value of between 10 and 30 degrees Celsius, wherein succinic acid cystals are formed. Preferably, the crystallizing comprises bringing the temperature of the concentrated solution between 15 and 25 degrees Celsius, preferably between 18 and 22 degrees Celsius. . Preferably, the aqueous solution has a pH of between 1.5 and 4.5, preferably between 2 and 4. The aqueous solution may be any suitable solution comprising succinic acid. The aqueous solution may comprise soluble constituents and insoluble constituents and, such as (fragments of) microbial cells , protein, plant biomass lignocellulose, cellulose and the like. Preferably the aqueous solution is a fermentation medium, preferably a fermentation medium obtainable by a process for the production of a dicarboxylic acid as described herein.

Genetic modifications

Standard genetic techniques, such as overexpression of enzymes in the host cells, genetic modification of host cells, or hybridisation techniques, are known methods in the art, such as described in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, *Cold Spring Harbor Laboratory Press*, or F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987). Methods for transformation, genetic modification etc of fungal host cells are known from e.g. EP-A-0 635 574, WO 98/46772, WO 99/60102 and WO 00/37671, WO90/14423, EP-A-0481008, EP-A-0635 574 and US 6,265,186.

The following examples are for illustrative purposes only and are not to be construed as limiting the invention.

EXAMPLES

Example 1. Construction yeast strain

1.1. Construction of expression constructs

The expression construct pGBS414PEK-2 was created after a *Bam*HI/*Not*I restriction of the *S. cerevisiae* expression vector pRS414 (Sirkoski R.S. and Hieter P, Genetics, 1989, 122(1):19-27) and subsequently ligating in this vector a *Bam*HI/*Not*I restriction fragment consisting of the phosphoenolpyruvate carboxykinase (origin *Mannheimia succiniciproducens*) synthetic gene construct (SEQ ID NO: 1). The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS414PEK-1. Subsequently, pGBK414PEK-1 was restricted with *Asc*I and *Not*I. To create pGBS414PEK-2, an *Asc*I/*Not*I restriction fragment consisting of mitochondrial fumarate reductase from *T. brucei* (FRDm1) synthetic gene construct (SEQ ID NO: 2) was ligated into the restricted pGBS414PEK-1 vector. The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS414PEK-2.

The expression construct pGBS415FUM-2 was created after a *Bam*HI/*Not*I restriction of the *S. cerevisiae* expression vector pRS415 (Sirkoski R.S. and Hieter P, Genetics, 1989, 122(1):19-27) and subsequently ligating in this vector a *Bam*HI/*Not*I restriction fragment consisting of the fumarase (origin *Rhizopus oryzae*) synthetic gene construct (SEQ ID NO: 3). The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS415FUM-1. Subsequently,

pGBK415FUM-1 was restricted with *AscI* and *NotI*. To create pGBS415FUM-2, an *AscI*/*Not*I restriction fragment consisting of modified cytoplasmic malate dehydrogenase from *S. cerevisiae* (delta12N MDH2) synthetic gene construct (SEQ ID NO: 4) was ligated into the restricted pGBS415FUM-1 vector. The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS415FUM-2.

The expression construct pGBS416MAE-1 was created after a *Bam*HI/*Not*I restriction of the *S. cerevisiae* expression vector pRS416 (Sirkoski R.S. and Hieter P, Genetics, 1989, 122(1):19-27) and subsequently ligating in this vector a *Bam*HI/*Not*I restriction fragment consisting of the *Schizosaccharomyces pombe* malate transporter synthetic gene construct (SEQ ID NO: 5). The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS416MAE-1.

1.2. Construction S. cerevisiae strain

Plasmids pGBS414PEK-2, pGBS415FUM-2 and pGBS416MAE-1 (described under 1.1.) were transformed into *S. cerevisiae* strain CEN.PK113-6B (*MATA ura3-52 leu2-112 trp1-289*) to create strain SUC-194, overexpressing PCKm, delta12NMDH2, FUMR, FRDm1 and SpMAE1. All genes were codon pair optimized for expression in *S. cerevisiae* according to WO2008/000632.

The expression vectors were transformed into yeast by electroporation. The transformation mixtures were plated on Yeast Nitrogen Base (YNB) w/o AA (Difco) + 2% glucose.

Example 2. Anaerobic succinic acid and ethanol production at low pH

The *S. cerevisiae* yeast strain SUC-194 (MATA ura3-52 leu2-112 trp1-289, overexpressing SpMAE1, PCKm, delta12NMDH2, FUMR and FRDm1) was cultivated in shake-flask (2 x 300 ml) for 3 days at 30°C and 220 rpm. The medium was based on Verduyn, but modifications in carbon and nitrogen source were made as shown in Table 1-3.

Table 1. Preculture shake flask medium composition

Raw material	Concentration (g/l)
Galactose ($C_6H_{12}O_6$. H_2O)	20.0
Urea $(NH_2)_2CO$	2.3
KH_2PO_4	3.0
$MgSO_4$. $7H_2O$	0.5
Trace element solution[a]	1
Vitamin solution[b]	1

Table 2. [a]Vitamin solution

Component	Formula	Concentration (g/kg)
Biotin (D-)	$C_{10}H_{16}N_2O_3S$	0.05
Ca D(+) panthothenate	$C_{18}H_{32}CaN_2O_{10}$	1.00
Nicotinic acid	$C_6H_5NO_2$	1.00
Myo-inositol	$C_6H_{12}O_6$	25.00
Thiamine chloride hydrochloride	$C_{12}H_{18}Cl_2N_4OS$. xH_2O	1.00
Pyridoxol hydrochloride	$C_8H_{12}ClNO_3$	1.00
p-aminobenzoic acid	$C_7H_7NO_2$	0.20

Table 3. [b]Trace elements solution

Component	Concentration (g/kg)
EDTA ($C_{10}H_{14}N_2Na_2O_8$. $2H_2O$)	15.00
$ZnSO_4.7H_2O$	4.50
$MnCl_2$. $2H_2O$	0.84
$CoCl_2$. $6H_2O$	0.30
$CuSO_4$. $5H_2O$	0.30
Na_2MoO_4 . $2H_2O$	0.40
$CaCl_2$. $2H_2O$	4.50
$FeSO_4.7H_2O$	3.00
H_3BO_3	1.00
KI	0.10

Subsequently, the content of the shake-flasks was transferred to 10L fermenter (Startweight 6 kg), which contained the medium with the composition shown in Table 4:

Table 4. Composition medium in fermenter

Raw material	Concentration (g/l)
$(NH_4)_2SO_4$	2.5
KH_2PO_4	3.0
$MgSO_4$. $7H_2O$	0.5
Trace element solution[b]	1
Vitamin solution[a]	1

During the first 24 hours of the fermentation aerobic conditions (0.33 vvm gasflow, controlled OUR of 30 mmol/h and 10% CO_2) were applied to generate sufficient biomass. The pH was controlled at 5.0 by addition of 6 N KOH. The temperature was controlled at 30°C. Glucose concentration was kept limited by controlled feed to the fermenter.

After 24 hours the conditions were switched to anaerobic conditions (0.33 vvm, 10% CO_2 and 90% N_2) and pH control was released.

2.1. NMR analyses

Dicarboxylic acid concentrations in the fermentation supernatant were determined by means of NMR spectroscopy.

3 ml broth was centrifuged for 10 min at 4500 x g. Approximately 500 microlitres of supernatant were accurately weighed to a headspace vial. To each sample 0.5 ml of pen buffer C-2696 (containing 5.62 mg/ml maleic acid) was added. The samples were capped and cooked for about 10 minutes in a water bath (and in oil bath in the control sample CF292706-11 and CF292706-12) at 100 °C. The samples were lyophilized, the residue was dissolved in 1 ml D_2O.

The spectra were recorded at a proton frequency of Bruker DRX 360 MHz at a probe temperature of 300 K. The quantitative measurements were performed with pulse program zg, excitation pulse from 30 - 90 degrees and a relaxation delay of 40s.
Ethanol and glycerol concentrations were measured using flow-NMR. To 900 microlitres supernatant 100 microlitres of 20 g/l maleic acid in D_2O containing 40 g/l EDTA were added. The samples were homogenized and measured with an Avance II 500 MHz BEST NMR, equipped with a cryprobe, operating at 500 MHz and temperature of 27 °C. The pulse program noesygppr1d.comp was used with relaxation delay of 30 s, ns = 4, ds = 0, and water suppression power level pl9 = 65 dB.

During the anaerobic cultivation the pH decreased to 3.25 after 90 hours of cultivation. After this period 19.5 g/L succinic acid, 25.1 g/L ethanol and minimal levels of glycerol and malic acid were produced (both 0.5 g/L).

Example 3. Recovery of succinic acid crystals from fermentation broth

3.1.

The broth obtained from the anaerobic fermentation process as described in example 2 was filtered on a pressure filter (Pall Z200 filter and subsequent Pall Z2000 filter). The obtained clear filtrate was concentrated by water evaporation at 80°C under mild vacuum until crystallization on-set was observed (visually). The concentrate was cooled to 20 °C, yielding succinic acid crystals. The slurry was filtered on a glass filter (Nr 2 standard glass filter) and the obtained succinic acid wet cake was washed with 1 bed volume of cold (5°C) demineralized water. The washed cake was dried in a vacuum stove for 24 hours.
NMR analysis indicated a purity in excess of 98% w/w in succinic acid on dry weight basis. The recovery yield of succinic acid was in excess of 80%.

3.2.

A second fermentation broth from anaerobic yeast fermentation as described in Example 2 was collected. The broth pH was 3.1. The broth was centrifuged at 4000G for 10 minutes to remove suspended biomass. The supernatant was collected and filtered on a polish filter (Pall Z2000 and Pall Z200) to remove residual suspended solids. A total of 5.5 kg of filtrate was collected, containing 15.7 gram succinic acid per liter. The filtrate was concentrated by a factor 16 by evaporation under mild vacuum (80 °C). During concentration residual traces of precipitate which were removed by filtration on filter cloth. Analysis indicated that the composition of the precipitate was >95% calcium sulphate.

The filtered concentrate was subsequently cooled to 20 °C, allowing the succinic acid to crystallize. The crystals in the slurry were recovered by filtration on filter cloth. The recovered crystals were washed with 1 bed volume of demineralized water and dried. A total of 53 gram of crystals was recovered, corresponding to a recovery yield of 61% w/w. Proton NMR analysis as described in Example 2.1. indicated that the crystals contained >99.3% w/w of succinic acid on dry weight basis.

The combined mother liquor (215 gram, pH 3.5), spent wash water (86 gram, pH 3.1) and equipment rinse water were collected and subsequently concentrated by evaporation and subsequently cooled to 20 °C as described above. A second crop of crystals was recovered and washed with 1 bed volume of demineralised water. The second crop of crystals (11.6 gram on dry weight) contained over 96% w/w succinic acid on dry weight basis.

The total recovery yield of both crops was calculated at 74% with an average purity of 99%.

The results in Example 3 show that it was possible to obtain high purity succinic acid crystals in a high amount in a simple and economical one-step crystallization process which did not require the addition of salts.

Example 4. Recovery of succinic acid from complex medium

500 ml of a model solution of a dry-grind bioethanol refinery of thin stillage as described by Kim *et al.* (Bioresource Technology 99 (2008), pages 5165-5176) was spiked with succinic acid. The pH of the stillage was between 3 and 4. The solids in the thin stillage were filtered off by pressure filtration (Pall Z200 and Z2000 pressure filtration), yielding a clear filtrate. The filtrate was concentrated by evaporation of water at 80 °C under mild vacuum, until the liquid became turbid (determined visually). The hot liquid was filtered (Pall Z200 filter) at 80 °C to remove precipitated substances and subsequently cooled. The slurry was filtered over a glass filter (nr 2 standard glass filter). The obtained filter cake was washed with 1 bed volume of cold (5°C) demineralized water. The obtained crystals were dried for 24h in a vacuum stove. NMR analysis of the dried crystals indicated a purity in excess of 98% w/w in succinic acid. The yield in was ~50% in succinic acid.

CLAIMS

1. Process for the production of a dicarboxylic acid and ethanol which comprises fermenting a genetically modified yeast in a suitable fermentation medium under anaerobic conditions at a pH value of between 1 and 5 and producing the dicarboxylic acid and ethanol.

2. Process according to claim 1 wherein the amount of dicarboxylic acid produced is at least 1 g/l.

3. Process according to claim 1 or 2, wherein the dicarboxylic acid is malic acid, fumaric acid or succinic acid.

4. Process according to any one of the claims 1 to 3 wherein the amount of ethanol produced is below 100 g / l.

5. Process according to any one of the claims 1 to 4, wherein the process comprises a step of preculturing the genetically modified yeast under aerobic conditions in the presence of a carbohydrate.

6. Process according to anyone of the claims 1 to 5, wherein the yeast expresses a nucleotide sequence encoding a phosphoenol pyruvate carboxykinase in the cytosol.

7. Process according to any one of the claims 1 to 6, wherein the yeast expresses a nucleotide sequence encoding a malate dehydrogenase in the cytosol.

8. Process according to any one of the claims 1 to 7, wherein the yeast expresses a nucleotide sequence encoding a fumarase in the cytosol.

9. Process according to anyone of the claims 1 to 8, wherein the yeast expresses a nucleotide sequence encoding a fumarate reductase in the cytosol.

10. Process according to any one of the claims 1 to 9, wherein the yeast expresses a nucleotide sequence encoding a malic acid transporter protein in the cytosol.

11. Process according to any one of the claims 1 to 10, further comprising recovering the dicarboxylic acid and / or ethanol.

12. Process according to claim 12, characterized in that the dicarboxylic acid is succinic acid and wherein the recovering comprises crystallizing succinic acid from a fermentation medium having a pH of between 1 and 5 and comprising succinic acid, comprising removing part of the fermentation medium by evaporation to obtain a concentrated solution, and bringing the temperature of the concentrated solution to a value of between 5 and 35 degrees Celsius, wherein succinic acid cystals are formed.

13. Process according to claims 11 or 12, further comprising using the dicarboxylic acid and / or ethanol in an industrial process.

14. Process according to any one of the claims 1 to 13, wherein the process is carried out on an industrial scale.

15. Process for producing a fermentation product by a genetically modified yeast, wherein ethanol production in the yeast is used to generate energy in the form of ATP under anaerobic conditions and a pH of between 1 and 5 in a suitable fermentation medium, and wherein the yeast comprises a genetic modification that increases the production of the fermentation product as compared to a yeast not comprising said genetic modification.

16. Process according to claim 15, wherein the fermentation product is a dicarboxylic acid.

17. Process for crystallizing succinic acid from an aqueous solution having a pH of between 1 and 5 and comprising succinic acid, comprising removing part of the

aqueous solution by evaporation to obtain a concentrated solution, and bringing the temperature of the concentrated solution to a value of between 10 and 30 degrees Celsius, wherein succinic acid cystals are formed.

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/053753

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/46
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

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, FSTA, BIOSIS, 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.
A	WO 2008/128522 A2 (ORGANO-BALANCE GMBH) 30 October 2008 (2008-10-30) * See page 20 (lines 4-17), pages 21-23 (gene modifications - compare present Claims 7-9), and pages 24-25 (yeasts) *	1-16
A	WO 2007/061590 A1 (TATE & LYLE INGREDIENTS AMERICAS, INC) 31 May 2007 (2007-05-31) cited in the application * See page 17 (pH 5) *	1-16

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[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 document 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)

"O" document referring to an oral disclosure, use, exhibition or other means

"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
13 July 2010	27/07/2010

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/053753

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OTERO ET AL: "Metabolic engineering of Saccharomyces cerevisiae microbial cell factories for succinic acid production" JOURNAL OF BIOTECHNOLOGY (SUPPLEMENT), vol. 131S, 2007, page S205, XP002544838 * See Abstract, the actual oral disclosure may have been more detailed *	1-16
A	CAMARASA ET AL: "Role in anaerobiosis of the isoenzymes for Saccharomyces cerevisiae fumarate reductase encoded by OSM1 and FRDS1" YEAST, vol. 24, 2007, pages 391-401, XP002591397 * See pages 392-393 (Materials and methods), pages 394-395 (Analytical methods), and pages 398-400 (Discussion) *	1-16
A	DE JONGH: "Organic acid production by Aspergillus niger / Chapter 6: Efforts towards engineering a succinate producing A. niger strain" 2005, BioCentrum - DTU / TECHNICAL UNIVERSITY OF DENMARK , XP002591518 * See the whole chapter / PhD Thesis *	1-16
A	MOON ET AL: "Metabolic engineering of Escherichia coli for the production of malic acid" BIOCHEMICAL ENGINEERING JOURNAL, vol. 40, 2008, pages 312-320, XP022680351 * See page 312 (Abstract / PEP) *	1-16
A	WU ET AL: "Enhanced anaerobic succinic acid production by Escherichia coli NZN111 aerobically grown on gluconeogenic carbon sources" ENZYME AND MICROBIAL TECHNOLOGY, vol. 44, March 2009 (2009-03), pages 165-169, XP025770366 * See page 165 (Introduction) *	1-16
A	DATABASE Geneseq [Online] 19 May 2005 (2005-05-19), HONG ET AL: "M. succiniciproducens protein" XP002544596 Database accession no. ADY72725 * Compare SEQ.ID.NO. 6: 99.8% identity in 538 aa. overlap (1-538) : (1-538); the mismatch at position 278 (N/F) *	1-16

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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.
A	DATABASE Geneseq [Online] 4 September 2008 (2008-09-04), PETERS ET AL: "Trypanosoma brucei fumarate reductase protein sequence, SEQ ID 10" XP002544597 Database accession no. ASQ26896 * Compare SEQ.ID.NO. 7: 100% identity in 1163 aa overlap (2-1164/Application) : (70-1232/'996) * -----	1-16
A	DATABASE EPO Proteins [Online] 17 December 2008 (2008-12-17), PUZIO ET AL: "Sequence 9800 from Patent WO2008142034" XP002544598 Database accession no. GM962846 * Compare SEQ.ID.NO. 8: 100% identity in 471 aa overlap (2-472/Application) : (24-494/'034) * -----	1-16
A	DATABASE Geneseq [Online] 2 April 2009 (2009-04-02), DE HULSTER ET AL: "Saccharomyces cerevisiae malate dehydrogenase, SEQ ID 13" XP002544599 Database accession no. AWF81162 * Compare SEQ.ID.NO. 9: 100% identity in 364 aa overlap (2-365/Application) : (14-377/'974) * -----	1-16
A	DATABASE Geneseq [Online] 15 June 2007 (2007-06-15), GROBLER ET AL: "Malate permease" XP002544600 Database accession no. AAW06355 * Compare SEQ.ID.NO. 10: 100% identity in 438 aa overlap (1-438) : (1-438) * -----	1-16
A	HUH ET AL: "Effective purification of succinic acid from fermentation broth produced by Mannheimia succiniciproducens" PROCESS BIOCHEMISTRY, vol. 41, 2006, pages 1461-1465, XP025124810 * See pages 1461-1462 (crystallization) and pages 1463-1464 (section 3.2) * -----	17

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/053753

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SONG ET AL: "Recovery of succinic acid produced by fermentation of a metabolically engineered Mannheimia succiniciproducens strain" JOURNAL OF BIOTECHNOLOGY, vol. 132, 2007, pages 445-452, XP022373169 * See pages 445-446 (Introduction) and 450-451 Discussion) *	17
A,P	WO 2009/065778 A1 (DSM IP ASSETS B.V.) 28 May 2009 (2009-05-28) * Related application by the same Applicant with overlapping subject-matter (see the whole document); SEQ.ID.NOS. 29, 34, 33, 31, and 40 are identical with SEQ.ID.NOS. 1-5 of the Application (NB: those sequences are not published, available online only). The international filing date is before the priority date of the Application *	1-16
A,P	WO 2009/065779 A1 (DSM IP ASSETS B.V.) 28 May 2009 (2009-05-28) * Related to the above citation; the international filing date is before the priority date of the Application *	1-16
A,P	WO 2009/101180 A2 (DSM IP ASSETS B.V.) 20 August 2009 (2009-08-20) * Related to the above citation; the internatinoal filing date is before the priority date of the Application *	1-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/053753

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

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

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purpose of search

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/053753

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008128522 A2	30-10-2008	DE 102007019184 A1	23-10-2008
		EP 2150619 A2	10-02-2010
		KR 20100017188 A	16-02-2010
WO 2007061590 A1	31-05-2007	AU 2006317058 A1	31-05-2007
		CA 2630333 A1	31-05-2007
		CN 101365782 A	11-02-2009
		EP 1954799 A1	13-08-2008
		JP 2009516526 T	23-04-2009
		KR 20080069678 A	28-07-2008
		US 2008090273 A1	17-04-2008
WO 2009065778 A1	28-05-2009	CA 2704654 A1	28-05-2009
		WO 2009065780 A1	28-05-2009
WO 2009065779 A1	28-05-2009	NONE	
WO 2009101180 A2	20-08-2009	NONE	