Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3023338 A1 2017/11/16

(21) 3 023 338

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2017/05/05
(87) Date publication PCT/PCT Publication Date: 2017/11/16
(85) Entrée phase nationale/National Entry: 2018/11/05
(86) N° demande PCT/PCT Application No.: EP 2017/060823
(87) N° publication PCT/PCT Publication No.: 2017/194424
(30) Priorité/Priority: 2016/05/10 (EP16305539.5)

(51) Cl.Int./Int.Cl. *C12P 7/18* (2006.01), *C12N 1/16* (2006.01), *C12N 9/04* (2006.01), *C12N 9/12* (2006.01)

(71) Demandeurs/Applicants:
INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE, FR;
UNIVERSITE LIBRE DE BRUXELLES, BE;
UNIVERSITE DE LIEGE, BE

(72) Inventeurs/Inventors:
NICAUD, JEAN-MARC, FR;
FICKERS, PATRICK, BE;
CARLY, FREDERIC, BE

(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : SOUCHES DE LEVURE MUTANTE A PRODUCTION AMELIOREE D'ERYTHRITOL OU D'ERYTHRULOSE
(54) Title: MUTANT YEAST STRAINS WITH ENHANCED PRODUCTION OF ERYTHRITOL OR ERYTHRULOSE

(57) Abrégé/Abstract:
The invention relates to a method for enhancing the erythritol and/or erythrulose productivity and/or yield of an erythritol and/or erythrulose-producing yeast strain, such as Yarrowia lipolytica, comprising inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase and/or erythritol dehydrogenase. The invention also relates to a mutant yeast strain obtained by said method.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 November 2017 (16.11.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/194424 A1

(51) International Patent Classification:
C12P 7/18 (2006.01) *C12N 9/12* (2006.01)
C12N 1/16 (2006.01) *C12N 9/04* (2006.01)

(21) International Application Number:
PCT/EP2017/060823

(22) International Filing Date:
05 May 2017 (05.05.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
16305539.5 10 May 2016 (10.05.2016) EP

(71) Applicants: **INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE** [FR/FR]; 147, rue de l'Université, 75007 PARIS (FR). **UNIVERSITE LIBRE DE BRUXELLES** [BE/BE]; ULB 50 avenue Franklin Roosevelt, 1050 BRUXELLES (BE). **UNIVERSITE DE LIEGE** [BE/BE]; 7 place du 20 août, 4000 LIEGE (BE).

(72) Inventors: **NICAUD, Jean-Marc**; 20 Avenue Hoche, 78190 TRAPPES (FR). **FICKERS, Patrick**; 52 quai de Rome, 4000 LIEGE (BE). **CARLY, Frederic**; Rue commandant Ponthier 1 bte 2, 1040 BRUXELLES (BE).

(74) Agent: **REGIMBEAU**; 139 rue Vendôme, 69477 LYON CEDEX 06 (FR).

(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, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, 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, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, 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, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, 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 2017/194424 A1

(54) Title: MUTANT YEAST STRAINS WITH ENHANCED PRODUCTION OF ERYTHRITOL OR ERYTHRULOSE

(57) Abstract: The invention relates to a method for enhancing the erythritol and/or erythrulose productivity and/or yield of an erythritol and/or erythrulose-producing yeast strain, such as *Yarrowia lipolytica*, comprising inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase and/or erythritol dehydrogenase. The invention also relates to a mutant yeast strain obtained by said method.

MUTANT YEAST STRAINS WITH ENHANCED PRODUCTION OF ERYTHRITOL OR ERYTHRULOSE

The present invention relates to mutant yeast strains, in particular mutant *Yarrowia* strains, having an enhanced erythritol and/or erythrulose production and/or yield. The present invention also relates to means and methods for obtaining these mutant yeast strains.

Erythritol is a four-carbon polyol naturally found in fruits, seaweeds or mushrooms, and produced by many osmophilic microorganisms as a protection against osmotic stress. In the food industry, erythritol is used as a food additive because of its sweetening properties. It is 60-70% as sweet as sucrose but it has low energy value, it is non-cariogenic and it does not affect glycemia. A large number of toxicological and clinical studies have shown its safety for human consumption, with no negative effect observed on health. It would also have antixodiant properties.

Industrially, erythritol is mainly produced by fermentation using osmophilic yeasts grown under high osmotic pressure. Most processes use glucose as a carbon source and are conducted either in batch or fed-batch fermentation mode (Moon *et al.*, 2010). Erythritol producer include *Aurobasidium sp.* (Ishizuka *et al.*, 1989), *Trigonopsis variabilis* (Kim *et al.*, 1997), *Torula sp.* (Lee *et al.*, 2000), *Candida magnoliae* (Ryu *et al.*, 2000) or *Pseudozyma tsubakaensis* (Jeya *et al.*, 2009) or *Yarrowia* (patent application EP 0 845 538).

Erythrulose (S-1,3,4-thihydroxy-2-butanone, L-glycero-2-tetrulose) is used in some self-tanning cosmetics, mostly in combination with dihydroxyacetone. Erythrulose reacts with amino acids from proteins of the stratum corneum and epidermis in a process similar to Maillard reaction. Erythrulose can also be used as a multifunctional chiron for the synthesis of polyoxygenated molecules such as macrolide and polyethers antibiotics.

Erythrulose can be obtained by chemical synthesis from formaldehyde and dihydroxyacetone by phosphate catalysis in neutral aqueous medium. It can also be synthesized using a transketolase catalysed reaction of lithium hydroxypyruvate and glycolaldehyde to erythrulose. A bioprocess of erythrulose synthesis from erythritol in

the bacteria *Gluconobacter frateurii* was reported in the literature (Moonmangnee *et al.*, 2002; Mizanur *et al.*, 2001).

Yarrowia lipolytica is a non-conventional dimorphic yeast, belonging to the subphylum Saccharomycotina. *Y. lipolytica* is well-known for its ability to use n-alkanes and fatty acids as carbon source, namely glucose, fructose and mannose (Barth and Gaillardin 1997; Nicaud 2012). Thanks to its ability to secrete high amounts of proteins and metabolites of interest, *Y. lipolytica* has been used in several industrial applications, including heterologuous protein production and citric acid production (Fickers *et al.*, 2005; Zinjarde, 2014). *Y. lipolytica* gave good results for erythritol production, and has the advantage of using raw glycerol as a carbon source instead of glucose (Rymowicz *et al.*, 2008). Raw glycerol, a byproduct of biodiesel production, is a renewable carbon source that it is both cheaper and more efficient than glucose for erythritol production (Tomaszewska *et al.*, 2012, Rywińska *et al.*, 2013).

Recently, *Yarrowia lipolytica*, in particular the acetate-negative mutant *Y. lipolytica* Wratislavia K1 (isolated from continuous citric acid fermentation with the parent strain of *Y. lipolytica* Wratislavia 1.31 in chemostat experiments) has been reported for erythritol production in fed-batch cultivations by using glycerol as the carbon source (Rymowicz *et al.*, 2008; Tomaszewska *et al.*, 2012). Carly *et al.* (2015) disclosed a genetically modified *Y. lipolytica* overexpressing glycerol kinase gene (GUT1) that showed a higher erythritol productivity.

The inventors have identified an essential gene of the erythritol catabolism in *Y. lipolytica,* YALI0F01606g, which encodes the protein referred to as SEQ ID NO: 1. They demonstrated that the loss of this gene is sufficient to remove the ability of *Y. lipolytica* to grow on erythritol. Although annotated as a dihydroxyacetone kinase, the properties of this gene indicate that it might code for an L-erythrulose kinase (EYK), an enzyme of the erythritol catabolism pathway, responsible for the conversion of L-erythrulose into L-erythrulose phosphate. To the knowledge of the inventors, it is the first EYK sequence (i.e. YALI0F01606g, gene *EYK1*) known in a living organism. Regardless of this, the results clearly showed that disrupting the YALI0F01606g gene have a positive effect on erythritol productions. A *Y. lipolytica* strain disrupted in the YALI0F01606g gene (FCY001 strain) displayed a higher yield of at least 25%, in particular from 25% to 35%, and a higher specific productivity of about 30% than the

wild-type strain W29. Even more, unlike the wild-type strain, erythritol concentration remained stable in the medium over time, making it a well-suited strain for industrial production without erythritol re-consumption. Further, said FCY001 strain is able to produce erythrulose in high biomass and high erythritol concentration conditions.

Accordingly, the present invention provides a method for enhancing the erythritol or erythrulose productivity and/or yield (advantageously the erythritol or erythrulose productivity and yield) of an erythritol and/or erythrulose-producing yeast strain, wherein said method comprises inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase (EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1).

L-erythrulose kinase (EC 2.7.1.27) belongs to the family of transferases, specifically those transferring phosphorus-containing groups (phosphotransferases) with an alcohol group as acceptor. The systematic name of this enzyme class is ATP:erythritol 4-phosphotransferase. This enzyme is also called erythritol kinase (phosphorylating). It catalyses the following reaction which requires ATP:

ATP + erythritol $\rightleftharpoons$ ADP + D-erythritol 4-phosphate

Methods for determining whether an enzyme has an L-erythrulose kinase (EC 2.7.1.27) activity are known in the art. By way of example, one can use the method described in Wu (2011).

In all the aspects of the present invention, the L-erythrulose kinase (EC 2.7.1.27) is preferably of sequence SEQ ID NO: 1.

In a preferred embodiment, the L-erythrulose kinase comprises or consists of the consensus amino acid sequence SEQ ID NO: 2. This sequence SEQ ID NO: 2 corresponds to the consensus amino acid sequence obtained by aligning the L-erythrulose kinase from the strains *Yarrowia lipolytica* CLIB122 (YALI_EYK1 of SEQ ID NO: 1), *Yarrowia galli* CBS 9722 (YAGA_ EYK1 of SEQ ID NO: 3), *Yarrowia yakushimensis* CBS 10253 (YAYA_ EYK1 of SEQ ID NO: 4), *Yarrowia alimentaria* CBS 10151 (YAAL_ EYK1 of SEQ ID NO: 5) and *Yarrowia phangnensis* CBS 10407 (YAPH_ EYK1of SEQ ID NO: 6).

The L-erythrulose kinase of SEQ ID NO: 3 (YAGA_ EYK1), SEQ ID NO: 4 (YAYA_ EYK1), SEQ ID NO: 5 (YAAL_ EYK1) and SEQ ID NO: 6 (YAPH_ EYK1) have respectively 96.77%, 91.62%, 87.22% and 85.01% identity with the polypeptide of sequence SEQ ID NO: 1 (YALI_ EYK1).

Unless otherwise specified, the percent of identity between two protein sequences which are mentioned herein is calculated from the BLAST results performed either at the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or at the GRYC (http://gryc.inra.fr/) websites using the BlastP program with the default BLOSUM62 parameters as described in Altschul *et al.* (1997).

Advantageously, if the yeast strain is a *Yarrowia* strain, the L-erythrulose kinase is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6.

The L-erythrulose kinase from the strain *Y. lipolytica* CLIB122 (YALI_EYK1) of SEQ ID NO: 1 is encoded in *Y. lipolytica* by the gene YALI0F01606g.

The erythritol and/or erythrulose -producing yeast strain (*i.e.*, a yeast strain capable of producing erythritol and/or erythrulose) include osmophilic yeast strains, which are capable of growing in media with high osmotic pressure, *i.e.*, in the presence of high sugar or salts concentration (see Moon *et al.*, 2010). They generally belong to the genus selected from the group consisting of *Aurobasidium*, *Candida*, *Moniliella* (or *Trichosporonoides*), *Pseudozyma*, *Torula*, *Trichosporon*, *Trigonopsis* or *Yarrowia*. More specifically, examples include *Aureobasidium sp.*, *Candida magnolia*, *Moniliella sp.*, *Moniliella tomentosa var. pollinis*, *Pseudozyma tsubakaensis*, *Torula sp*, *Trichosporon sp.*, *Trigonopsis variabilis*, *Yarrowia sp.*, *Yarrowia alimentaria Yarrowia galli, Yarrowia lipolytica, Yarrowia phangnensis* and *Yarrowia yakushimensis*.

In a preferred embodiment, the erythritol and/or erythrulose-producing yeast strain is a *Yarrowia* strain, more preferably is selected from the group consisting of *Y. lipolytica*, *Y. galli*, *Y. yakushimensis*, *Y. alimentaria* and *Y. phangnensis*, most preferably is a *Y. lipolytica* strain.

Said *Yarrowia* strain can be auxotrophic for leucine (Leu-) and optionally for the decarboxylase orotidine-5'-phosphate (Ura-).

Advantageously, the erythritol and/or erythrulose-producing yeast strain is selected from the group consisting of *Y. lipolytica*, *Y. galli*, *Y. yakushimensis*,

Y. alimentaria and *Y. phangnensis* and the L-erythrulose kinase is respectively selected from the group consisting of SEQ ID NO: 1, 3, 4, 5 and 6.

The method for enhancing the erythritol or erythrulose productivity and/or yield of an erythritol and/or erythrulose-producing yeast strain according to the present invention can further comprises overexpressing in said strain at least one gene encoding enzyme involved in the pathway of erythritol biosynthesis and/or at least one gene encoding enzyme involved in the pathway of erythrulose biosynthesis and/or inhibiting the expression or activity of at least one endogenous gene involved in erythritol catabolism.

Enzymes involved in the pathway of erythritol biosynthesis are described in Moon *et al.*, 2010. Advantageously, said enzyme involved in the pathway of erythritol biosynthesis is selected from the group consisting of:

- a glycerol kinase (EC 2.7.1.30), advantageously a yeast glycerol kinase, more advantageously an endogenous glycerol kinase of said strain,
- a glycerol-3P dehydrogenase (EC 1.1.5.3), advantageously a yeast glycerol-3P dehydrogenase, more advantageously an endogenous glycerol-3P dehydrogenase of said strain,
- a triose isomerase (EC 5.3.1.1), advantageously a yeast triose isomerase, more advantageously an endogenous triose isomerase of said strain,
- a transketolase (EC 2.2.1.1), advantageously a yeast transketolase, more advantageously an endogenous transketolase of said strain,
- an erythrose 4 phosphate phosphatase (EC 3.1.3.23), such as an erythrose 4 phosphate phosphatase corresponding to the enzyme named erythrose-4-phosphatase in Kuznetsova *et al.* (2006) or erythrose-4-phosphate phosphatase in Moon *et al.* (2010), advantageously an endogenous erythrose 4 phosphate phosphatase of said strain,
- an erythrose reductase (EC 1.1.1.21), advantageously a yeast erythrose reductase, more advantageously an endogenous erythrose reductase of said strain, and
- an invertase (EC 3.2.1.26), advantageously a yeast invertase, more advantageously the *S cerevisiae* invertase.

More advantageously, said enzyme involved in the pathway of erythritol biosynthesis is a glycerol kinase as defined above and/or a transketolase as defined

above, and even more advantageously the enzymes involved in the pathway of erythritol biosynthesis are a glycerol kinase as defined above and a transketolase as defined above.

Advantageously, said enzyme involved in the pathway of erythrulose biosynthesis is selected from the group consisting of:

- a glycerol kinase (EC 2.7.1.30), advantageously a yeast glycerol kinase, more advantageously an endogenous glycerol kinase of said strain,

- a glycerol-3P dehydrogenase (EC 1.1.5.3), advantageously a yeast glycerol-3P dehydrogenase, more advantageously an endogenous glycerol-3P dehydrogenase of said strain,

- a triose isomerase (EC 5.3.1.1), advantageously a yeast triose isomerase, more advantageously an endogenous triose isomerase of said strain,

- a transketolase (EC 2.2.1.1), advantageously a yeast transketolase, more advantageously an endogenous transketolase of said strain,

- an erythrose 4 phosphate phosphatase (EC 3.1.3.23), such as an erythrose 4 phosphate phosphatase corresponding to the enzyme named erythrose-4-phosphatase in Kuznetsova *et al.* (2006) or erythrose-4-phosphate phosphatase in Moon *et al.* (2010), advantageously an endogenous erythrose 4 phosphate phosphatase of said strain,

- an erythrose reductase (EC 1.1.1.21), advantageously a yeast erythrose reductase, more advantageously an endogenous erythrose reductase of said strain,

- an invertase (EC 3.2.1.26), advantageously a yeast invertase, more advantageously the *S cerevisiae* invertase, and

- an erythritol dehydrogenase (EC 1.1.1.9), such as an erythritol dehydrogenase described in Paradowska and Nitka (2009), advantageously a yeast erythritol:NAD+ 2-oxydoreductase or more precisely a yeast erythritol dehydrogenase, more advantageously an endogenous erythritol:NAD+ 2-oxydoreductase of said strain or more precisely a yeast erythritol dehydrogenase of said strain.

More advantageously, said enzyme involved in the pathway of erythrulose biosynthesis is an erythritol dehydrogenase as defined above, and even more advantageously the enzymes involved in the pathway of erythrulose biosynthesis are an erythritol dehydrogenase as defined above and a glycerol kinase as defined above and/or a transketolase as defined above, and even more advantageously the enzymes involved in the pathway of erythrulose biosynthesis are an erythritol dehydrogenase as

defined above and a glycerol kinase as defined above and a transketolase as defined above.

Advantageously, said enzyme involved in the pathway of erythritol catabolism, in particular in bioconversion of erythritol into erythrulose, is an erythritol dehydrogenase (EC 1.1.1.9), such as an erythritol dehydrogenase described in Paradowska and Nitka (2009), advantageously a yeast erythritol:NAD+ 2-oxydoreductase or more precisely a yeast erythritol dehydrogenase, more advantageously an endogenous erythritol:NAD+ 2-oxydoreductase of said strain or more precisely a yeast erythritol dehydrogenase of said strain.

Erythritol dehydrogenase (EC 1.1.1.9) belongs to the family of oxidoreductase, specifically to polyol deshydrogenase, more specifically erythritol deshydrogenase. The systematic name of this enzyme class is erythritol:NAD+ 2-oxydoreductase. It catalyses the oxidation of erythritol into erythulose following reaction: erythritol + NAD $\rightleftharpoons$ erythrulose + NADH + H.

Methods for determining whether an enzyme has an activity of erythritol dehydrogenase (EC 1.1.1.9) are known in the art. By way of example, one can use the method described in Paradowska and Nitka (2009).

In all the aspects of the present invention, the erythritol dehydrogenase (EC 1.1.1.9) is preferably of sequence SEQ ID NO: 7.

The inhibition of the expression or activity of the endogenous L-erythrulose kinase or of the endogenous erythritol dehydrogenase can be total or partial. It may be obtained in various ways by methods known in themselves to those skilled in the art. The term inhibiting the expression or activity of an endogenous L-erythrulose kinase or of an erythritol dehydrogenase in a yeast strain refers to decreasing the quantity of said enzyme produced in a yeast strain compared to a reference (control) yeast strain wherein the expression or activity of said endogenous L-erythrulose kinase or of said endogenous erythritol dehydrogenase is not inhibited and from which the mutant strain derives.

This inhibition may be obtained by mutagenesis of the endogenous gene encoding said L-erythrulose kinase (*EYK1* gene) or said erythritol dehydrogenase (*EYD1* gene) using recombinant DNA technology or random mutagenesis. This may be obtained by

various techniques, performed at the level of DNA, mRNA or protein, to inhibit the expression or the activity of the L-erythrulose kinase or of the erythritol dehydrogenase.

At the level of DNA, mRNA, this inhibition may be accomplished by deletion, insertion and/or substitution of one or more nucleotides, site-specific mutagenesis, random mutagenesis, targeting induced local lesions in genomes (TILLING), knock-out techniques, or gene silencing using, *e.g.*, RNA interference, antisense, aptamers, and the like.

This inhibition may also be obtained by insertion of a foreign sequence in the *EYK1* gene or *EYD1* gene, *e.g.*, through transposon mutagenesis using mobile genetic elements called transposons, which may be of natural or artificial origin.

The mutagenesis of the endogenous gene encoding said L-erythrulose kinase (*EYK1* gene) or of the endogenous erythritol dehydrogenase can be performed at the level of the coding sequence or of the sequences for regulating the expression of this gene, in particular at the level of the promoter, resulting in an inhibition of transcription or of translation of said L-erythrulose kinase or said erythritol dehydrogenase.

The mutagenesis of the endogenous *EYK1* gene or of the endogenous *EYD1* gene can be carried out by genetic engineering. It is, for example, possible to delete all or part of said gene and/or to insert an exogenous sequence. Methods for deleting or inserting a given genetic sequence in yeast, in particular in *Y. lipolytica*, are well known to those skilled in the art (for review, see Barth and Gaillardin, 1996; Madzak *et al.*, 2004). By way of example, one can use the method referred to as POP IN/POP OUT which has been used in yeasts, in particular in *Y. lipolytica*, for deleting the *LEU2* and *XPR2* genes (Barth and Gaillardin, 1996). One can also use the SEP method (Maftahi *et al.*, 1996) which has been adapted in *Y. lipolytica* for deleting the *POX* genes (Wang *et al.*, 1999). One can also use the SEP/Cre method developed by Fickers *et al.* (2003) and described in International application WO 2006/064131. In addition, methods for inhibiting the expression or the activity of an enzyme in yeasts are described in International application WO 2012/001144.

An advantageous method according to the present invention consists in replacing the coding sequence of the endogenous *EYK1* gene or of the endogenous *EYD1* gene with an expression cassette containing the sequence of a gene encoding a selectable marker. It is also possible to introduce one or more point mutations into the endogenous

EYK1 gene or into the endogenous *EYD1* gene, resulting in a shift in the reading frame, and/or to introduce a stop codon into the sequence and/or to inhibit the transcription or the translation of the endogenous *EYK1* gene or of the endogenous *EYD1* gene.

Another advantageous method according to the present invention consists in genetically transforming said yeast strain with a disruption cassette of said endogenous *EYK1* gene or of said endogenous *EYD1* gene. A suitable disruption cassette for disrupting the endogenous *EYK1* gene or the endogenous *EYD1* gene contains specific sequences for homologous recombination and site-directed insertion, and a selection marker.

The mutagenesis of the endogenous *EYK1* gene or of the endogenous *EYD1* gene can also be carried out using physical agents (for example radiation) or chemical agents. This mutagenesis also makes it possible to introduce one or more point mutations into the *EYK1* gene or into the *EYD1* gene.

The mutated *EYK1* gene or the mutated *EYD1* gene can be identified for example by PCR using primers specific for said gene.

It is possible to use any selection method known to those skilled in the art which is compatible with the marker gene (or genes) used. The selectable markers which enable the complementation of an auxotrophy, also commonly referred to as auxotrophic markers, are well known to those skilled in the art in the field of yeast transformation. The URA3 selectable marker is well known to those skilled in the art. More specifically, a yeast strain in which the *URA3* gene (sequence available in the Génolevures database (http://genolevures.org/) under the name YALI0E26741g or the UniProt database under accession number Q12724), encoding orotidine-5'-phosphate decarboxylase, is inactivated (for example by deletion), will not be capable of growing on a medium not supplemented with uracil. The integration of the URA3 selectable marker into this yeast strain will then make it possible to restore the growth of this strain on a uracil-free medium. The LEU2 selectable marker described in particular in patent US 4 937 189 is also well known to those skilled in the art. More specifically, a yeast strain in which the *LEU2* gene (*e.g.*, YALI0C00407g in *Y. lipolytica*), encoding β-isopropylmalate dehydrogenase, is inactivated (for example by deletion), will not be capable of growing on a medium not supplemented with leucine. As previously, the integration of the LEU2 selectable marker into this yeast strain will then make it

possible to restore the growth of this strain on a medium not supplemented with leucine. The ADE2 selectable marker is also well known to those skilled in the art. A yeast strain in which the *ADE2* gene (*e.g.*, YALI0B23188g in *Y. lipolytica*), encoding phosphoribosylaminoimidazole carboxylase, is inactivated (for example by deletion), will not be capable of growing on a medium not supplemented with adenine. Here again, the integration of the ADE2 selectable marker into this yeast strain will then make it possible to restore the growth of this strain on a medium not supplemented with adenine. Leu$^-$ Ura$^-$ auxotrophic *Y. lipolytica* strains have been described by Barth and Gaillardin, 1996.

In a preferred embodiment, the method for enhancing the erythritol productivity and/or yield of an erythritol-producing yeast strain comprises inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase (EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and overexpressing at least 1, 2, 3, 4, 5, 6 or the 7 enzymes selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase, preferably overexpressing at least a glycerol kinase or a transketolase. More preferably, the method for enhancing the erythritol productivity and/or yield of an erythritol-producing yeast strain comprises inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase (EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and overexpressing a glycerol kinase and a transketolase.

In another preferred embodiment, the method for enhancing the erythrulose productivity and/or yield of an erythrulose -producing yeast strain comprises inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase (EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and overexpressing at least an erythritol dehydrogenase and optionally at least 1, 2, 3, 4, 5, 6

or the 7 enzymes selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, a fumarase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase. Preferably the method for enhancing the erythrulose productivity and/or yield of an erythrulose-producing yeast strain comprises inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase (EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and overexpressing an erythritol dehydrogenase and a glycerol kinase or a transketolase. More preferably, the method for enhancing the erythrulose productivity and/or yield of an erythrulose-producing yeast strain comprises inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase (EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and overexpressing an erythritol dehydrogenase, a glycerol kinase and a transketolase.

Preferably, said erythritol dehydrogenase is a polypeptide of sequence SEQ ID NO: 7 (YALI_EYD1) or an erythritol dehydrogenase having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 7 (YALI_EYD1).

In another aspect, the present invention is related to a method for enhancing the erythritol productivity and/or yield of an erythritol-producing yeast strain without production of erythrulose, said method comprising inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase (EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and inhibiting in said yeast strain the expression or the activity of an endogenous erythritol dehydrogenase (EC 1.1.1.9). Optionally said method comprises overexpressing at least 1, 2, 3, 4, 5, 6 or the 7 enzymes selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate

phosphatase, an erythrose reductase and an invertase, preferably overexpressing at least a glycerol kinase or a transketolase, preferably overexpressing a glycerol kinase and a transketolase. Preferably, said endogenous erythritol dehydrogenase is a polypeptide of sequence SEQ ID NO: 7 (YALI_EYD1) or an erythritol dehydrogenase having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 7 (YALI_EYD1).

In another aspect, the present invention is related to a method for enhancing the erythritol productivity and/or yield of an erythritol-producing yeast strain without production of erythrulose, said method comprising inhibiting in said yeast strain the expression or the activity of an endogenous erythritol dehydrogenase (EC 1.1.1.9) and optionally overexpressing in said strain at least one enzyme selected from the group consisting of a glycerol kinase (EC 2.7.1.30), a glycerol-3P dehydrogenase (EC 1.1.5.3), a triose isomerase (EC 5.3.1.1), a transketolase (EC 2.2.1.1), an erythrose 4 phosphate phosphatase (EC 3.1.3.23), an erythrose reductase (EC 1.1.1.21) and an invertase (EC 3.2.1.26), preferably a glycerol kinase and/or a transketolase, even more preferably a glycerol kinase and a transketolase. Preferably, said endogenous erythritol dehydrogenase is a polypeptide of sequence SEQ ID NO: 7 (YALI_EYD1) or an erythritol dehydrogenase having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 7 (YALI_EYD1).

Advantageously, in all the aspects of the present invention where a glycerol kinase and/or a transketolase is overexpressed, the glycerol kinase is encoded by the *GUT1* gene and/or the transketolase is encoded by the *TKL1* gene.

The enzyme(s) overexpressed in said yeast strain can be an endogenous enzyme of said strain. The enzyme(s) overexpressed in said yeast strain can also be from any prokaryotic or eukaryotic organism. The coding sequence of the genes encoding this/these enzyme(s) can be optimized for its expression in the yeast by methods well known to those skilled in the art (for review, see Hedfalk, 2012).

The term overexpressing an enzyme in a yeast strain, herein refers to artificially increasing the quantity of said enzyme produced in a yeast strain compared to a reference (control) yeast strain wherein said enzyme is not overexpressed. This term

also encompasses expression of an enzyme in a yeast strain which does not naturally contain a gene encoding said enzyme.

The glycerol kinase activity of an enzyme can be measured by quantifying formation of glyceroladehyde 3 phosphate from glycerol, as described in Sprague *et al.* (1977).

In yeasts, the glycerol kinase is encoded by the *GUT1* gene. More particularly, the coding sequence of the *GUT1* gene and the peptide sequence of the glycerol kinase of *Y. lipolytica* CLIB122 are available in the Génolevures or GenBank databases under the following accession numbers YALI0F00484g/YALI0F00484p (referred to as SEQ ID NO: 8).

The glycerol-3P dehydrogenase activity of an enzyme can be measured by quantifying the release of dihydroxyacetone phosphate from glycerol 3 phosphate, as described in Lindgren *et al.* (1977).

In yeasts, the glycerol-3P dehydrogenase is encoded by the *GUT2* gene. More particularly, the coding sequence of the *GUT2* gene and the peptide sequence of the glycerol-3P dehydrogenase of *Y. lipolytica* CLIB122 are available in the Génolevures or GenBank databases under the following accession numbers YALI0B13970g/YALI0B13970p (referred to as SEQ ID NO: 9).

The triose phosphate isomerase activity of an enzyme can be measured by quantifying the release of dihydroxyacetone phosphate from glyceraldehyde 3 phosphate, as described in Sharma *et al.* (2012).

In yeasts, the triose phosphate isomerase is encoded by the *TIM1* gene. More particularly, the coding sequence of the *TIM1* gene and the peptide sequence of the triose phosphate isomerase of *Y. lipolytica* CLIB122 are available in the Génolevures or GenBank databases under the following accession numbers YALI0F05214g/YALI0F05214p (referred to as SEQ ID NO: 10).

The transketolase activity of an enzyme can be measured by quantifying the formation of NAD+ from xylulose 5 phosphate, ribose 5 phosphate and NADH, as described in Matsushika *et al.* (2012).

In yeasts, the transketolase is encoded by the *TKL1* gene. More particularly, the coding sequence of the *TKL1* gene and the peptide sequence of the transketolase of *Y. lipolytica* CLIB122 are available in the Génolevures or GenBank databases under the

following accession numbers YALI0E06479g/YALI0E06479p (referred to as SEQ ID NO: 11).

The erythrose 4 phosphate phosphatase activity of an enzyme can be measured by quantifying the formation of erythrose from erythrose 4 phosphate. It could also be screened by the detection of released phosphate (Pi) with the highly sensitive Malachite Green reagent as described in Baykov *et al.* (1988) or Kuznetsova *et al.* (2006).

The erythrose 4 phosphate phosphatase is encoded by an *E4PK* gene. The yeast gene coding for this enzyme has not been yet identified. However in bacteria, some proteins have shown to present erythrose 4 phosphate phosphatase activity. In *Synechocys* sp PCC6803 the erythrose 4 phosphate phosphatase is encoded by the *sII1524* gene (Accession number WP_010873080 in the GeneBank database, International Application WO 2015/147644). In *Thermotoga maritima* MSB8 the erythrose 4 phosphate phosphatase is encoded by the TM1254 gene (Accession number NP_229059 in the GeneBank database, International Application WO 2015/147644). In *Escherichia coli* strain K12 the erythrose 4 phosphate phosphatase is encoded by the YidA gene (Accession number NP_418152 in the GeneBank database (Kuznetsova *et al.*, 2006)).

The erythrose reductase activity of an enzyme can be measured by quantifying the formation of NADP+ from erythrose and NADPH, as described in Ishizuka *et al.* (1992).

In yeasts, the erythrose reductase is encoded by a gene belonging to the aldo-keto reductase family (AKR or ALR). The coding sequence of the *AKR* gene and the amino acid sequence of the erythrose reductase of *Candida magnolia* (ALR1) are available in the GenBank database under the following accession number FJ550210 (Lee *et al.*, 2010, referred to as SEQ ID NO: 12).

The invertase activity of an enzyme can be measured by quantifying the release of reducing sugar from sucrose as described in Miller (1959).

A genetically modified *Y. lipolytica* strain comprising an invertase expression cassette composed of *Saccharomyces cerevisiae* Suc2p secretion signal sequence followed by the *SUC2* sequence and under the control of the *Y. lipolytica* pTEF promoter is described in Lazar *et al.* (2013). The overexpression of invertase allows growth on sucrose-based raw materials.

Advantageously, the enzyme to overexpress is an endogenous enzyme of the mutated strain, provided that said strain naturally expresses the enzyme as defined above.

Overexpression of an enzyme as defined above - which can be an endogenous, ortholog or heterologous enzyme - in a yeast strain, in particular in a *Yarrowia* strain according to the present invention can be obtained in various ways by methods known *per se*.

Overexpression of an enzyme as defined in the present invention may be performed by placing one or more (preferably two or three) copies of the coding sequence (CDS) of the sequence encoding said enzyme under the control of appropriate regulatory sequences. Said regulatory sequences include promoter sequences, located upstream (at 5' position) of the ORF of the sequence encoding said enzyme, and terminator sequences, located downstream (at 3' position) of the ORF of the sequence encoding said enzyme.

Promoter sequences that can be used in yeast are well known to those skilled in the art and may correspond in particular to inducible or constitutive promoters. Examples of promoters which can be used according to the present invention, include the promoter of a *Y. lipolytica* gene which is strongly repressed by glucose and is inducible by the fatty acids or triglycerides such as the promoter of the *POX2* gene encoding the acyl-CoA oxidase 2 (AOX2) of *Y. lipolytica* and the promoter of the *LIP2* gene described in International Application WO 01/83773. One can also use the promoter of the *FBA1* gene encoding the fructose-bisphosphate aldolase (see Application US 2005/0130280), the promoter of the *GPM* gene encoding the phosphoglycerate mutase (see International Application WO 2006/0019297), the promoter of the *YAT1* gene encoding the transporter ammonium (see Application US 2006/0094102), the promoter of the *GPAT* gene encoding the O-acyltransferase glycerol-3-phosphate (see Application US 2006/0057690), the promoter of the *TEF* gene (Müller *et al.*, 1998; Application US 2001/6265185), the hybrid promoter hp4d (described in International Application WO 96/41889), the hybrid promoter XPR2 described in Mazdak *et al.* (2000) or the hybrid promoters UAS1-TEF or UAStef-TEF described in Blazeck *et al.* (2011, 2013, 2014).

Advantageously, the promoter is the promoter of the *TEF* gene.

Terminator sequences that can be used in yeast are also well known to those skilled in the art. Examples of terminator sequences which can be used according to the present invention include the terminator sequence of the *PGK1* gene and the terminator sequence of the *LIP2* gene described in International Application WO 01/83773.

The nucleotide sequence of the coding sequences of the heterologous genes can be optimized for expression in yeast by methods well known in the art (see for review Hedfalk, 2012).

Overexpression of an endogenous enzyme as defined above can be obtained by replacing the sequences controlling the expression of said endogenous enzyme by regulatory sequences allowing a stronger expression, such as those described above. The skilled person can replace the copy of the gene encoding an endogenous enzyme in the genome, as well as its own regulatory sequences, by genetically transforming the yeast strain with a linear polynucleotide comprising the ORF of the sequence coding for said endogenous enzyme under the control of regulatory sequences such as those described above. Advantageously, said polynucleotide is flanked by sequences which are homologous to sequences located on each side of said chromosomal gene encoding said endogenous enzyme. Selection markers can be inserted between the sequences ensuring recombination to allow, after transformation, to isolate the cells in which integration of the fragment occurred by identifying the corresponding markers. Advantageously also, the promoter and terminator sequences belong to a gene different from the gene encoding the endogenous enzyme to be overexpressed in order to minimize the risk of unwanted recombination into the genome of the yeast strain.

Overexpression of an endogenous enzyme as defined above can also be obtained by introducing into the yeast strain extra copies of the gene encoding said endogenous enzyme under the control of regulatory sequences such as those described above. Said additional copies encoding said endogenous enzyme may be carried by an episomal vector, that is to say capable of replicating in the yeast strain. Preferably, these additional copies are carried by an integrative vector, that is to say, integrating into a given location in the yeast genome, *e.g.*, *Yarrowia* genome (Madzak *et al.*, 2004). In this case, the polynucleotide comprising the gene encoding said endogenous enzyme under the control of regulatory regions is integrated by targeted integration. Said additional copies can also be carried by PCR fragments whose ends are homologous to

a given locus of the yeast strain, allowing integrating said copies into the yeast genome by homologous recombination. Said additional copies can also be carried by auto-cloning vectors or PCR fragments, wherein the ends have a zeta region absent from the genome of the yeast, allowing the integration of said copies into the yeast genome, *e.g.*, *Yarrowia* genome, by random insertion as described in Application US 2012/0034652.

Targeted integration of a gene into the genome of a yeast cell is a molecular biology technique well known to those skilled in the art: a DNA fragment is cloned into an integrating vector, introduced into the cell to be transformed, wherein said DNA fragment integrates by homologous recombination in a targeted region of the recipient genome (Orr-Weaver *et al.*, 1981).

Methods for transforming yeast are also well known to those skilled in the art and are described, *inter alia*, by Ito *et al.* (1983), Klebe *et al.* (1983) and Gysler *et al.*, (1990).

Any gene transfer method known in the art can be used to introduce a gene encoding an enzyme. Preferably, one can use the method with lithium acetate and polyethylene glycol described by Gaillardin *et al.*, (1987) and Le Dall *et al.*, (1994).

A preferred method for overexpressing an enzyme in a yeast strain comprises introducing into the genome of said yeast strain a DNA construct comprising a nucleotide sequence encoding said enzyme, placed under the control of a promoter.

Method for overexpressing genes in *Yarrowia lipolytica* is well known as described in example in Nicaud *et al.* (2002) and Nicaud (2012).

The overexpression of *Y. lipolytica* endogenous *Y. lipolytica* genes *GUT1 GUT2*, *TKL1*, and the heterologous *Candida Magnoliae* cm*ALR1* gene in a *Y. lipolytica* strain is reported in Carly *et al.*, 2015.

The present invention also provides means for carrying out said overexpression.

This includes, in particular, recombinant DNA constructs for expressing at least one enzyme as defined above (GUT1, GUT2, TIM, TKL1, E4PK, ALR1, SUC2, EYD1) in a yeast cell, in particular in a *Yarrowia* cell. These DNA constructs can be obtained and introduced in said yeast strain by the well-known techniques of recombinant DNA and genetic engineering.

Recombinant DNA constructs of the invention include in particular expression cassettes, comprising a polynucleotide encoding at least one enzyme as defined above

(*i.e.*, a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase, an invertase, an erythritol dehydrogenase) preferably a glycerol kinase and/or a transketolase and/or an erythritol dehydrogenase, each polynucleotide encoding an enzyme being under the control of a promoter functional in a yeast cell as defined above.

The expression cassettes generally also include a transcriptional terminator, such as those describes above. They may also include other regulatory sequences, such as transcription enhancer sequences.

Recombinant DNA constructs of the invention also include recombinant vectors containing expression cassettes comprising a polynucleotide encoding at least one enzyme as defined above, each polynucleotide encoding an enzyme being under transcriptional control of a suitable promoter.

Recombinant vectors of the invention may also include other sequences of interest, such as, for instance, one or more marker genes, which allow for selection of transformed yeast cells.

The invention also comprises host cells containing a recombinant DNA construct of the invention. These host cells can be prokaryotic cells (such as bacteria cells) or eukaryotic cells, preferably yeast cells.

The invention also provides a method for obtaining a mutant erythritol-producing yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, having an enhanced erythritol productivity and/or yield (advantageously an enhanced erythritol productivity and yield) compared to the parent yeast strain, comprising inhibiting in the parent erythritol-producing yeast strain (of said mutant yeast strain) the expression or the activity of an endogenous L-erythrulose kinase (EYK; EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and optionally overexpressing in said yeast strain at least one enzyme selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase as defined above, preferably a glycerol kinase or a transketolase and more preferably a glycerol kinase and a transketolase.

Said overexpression can be obtained by transforming said yeast cell with at least one recombinant DNA constructs as defined above for expressing at least one enzyme selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase as defined above, preferably a glycerol kinase or a transketolase and more preferably a glycerol kinase and a transketolase.

More preferably, the method for obtaining a mutant erythritol-producing yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, having an enhanced erythritol productivity and/or yield (advantageously an enhanced erythritol productivity and yield) compared to the parent yeast strain, comprising inhibiting in the parent erythritol-producing yeast strain (of said mutant yeast strain) the expression or the activity of an endogenous L-erythrulose kinase (EYK; EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and overexpressing in said yeast strain a glycerol kinase and a transketolase.

More advantageously, for obtaining a mutant erythritol-producing yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, having an enhanced erythritol productivity and/or yield (advantageously an enhanced erythritol productivity and yield) compared to the parent yeast strain, comprising inhibiting in the parent erythritol-producing yeast strain (of said mutant yeast strain) the expression or the activity of an endogenous L-erythrulose kinase (EYK; EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and overexpressing in said yeast strain the glycerol kinase encoded by the *GUT1* gene and the transketolase encoded by the *TKL1* gene.

In one embodiment, the method for obtaining a mutant erythrulose-producing yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, having an enhanced erythrulose productivity and/or yield (advantageously an enhanced erythrulose productivity and yield) compared to the parent yeast strain, comprises inhibiting in the parent erythrulose-producing yeast strain (of said mutant

yeast strain) the expression or the activity of an endogenous L-erythrulose kinase (EYK; EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and overexpressing in said yeast strain an erythritol dehydrogenase and optionally overexpressing in said yeast strain at least one enzyme selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase as defined above, preferably a glycerol kinase and/or a transketolase. Preferably said method comprises overexpressing the erythritol dehydrogenase encoded by the *EYD1* gene and optionally the glycerol kinase encoded by the *GUT1* gene and/or the transketolase encoded by the *TKL1* gene.

Also in this aspect of the invention, the EYD1 gene is preferably of sequence SEQ ID NO: 7 (YALI_EYD1).

In another aspect, the present invention is also related to a method for obtaining a mutant erythritol-producing yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, having an enhanced erythritol productivity and/or yield (advantageously an enhanced erythritol productivity and yield) without production of erythrulose compared to the parent yeast strain, comprising inhibiting in the parent erythrulose-producing yeast strain (of said mutant yeast strain) the expression or the activity of an endogenous L-erythrulose kinase (EYK; EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1) and inhibiting the expression or the activity of an endogenous erythritol dehydrogenase, preferably inhibiting the expression or the activity of the endogenous erythritol dehydrogenase of sequence SEQ ID NO: 7 (YALI_EYD1) or of an endogenous erythritol dehydrogenase having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the sequence SEQ ID NO: 7 (YALI_EYD1). Optionally said method further comprises overexpressing in said yeast strain at least one enzyme selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4

phosphate phosphatase, an erythrose reductase and invertase as defined above, preferably a glycerol kinase and/or a transketolase. Preferably said method comprises overexpressing the glycerol kinase encoded by the *GUT1* gene and/or the transketolase encoded by the *TKL1* gene.

In another aspect, the present invention is also related to a method for obtaining a mutant erythritol-producing yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, having an enhanced erythritol productivity and/or yield (advantageously an enhanced erythritol productivity and yield) without production of erythrulose compared to the parent yeast strain, comprising inhibiting the expression or the activity of an endogenous erythritol dehydrogenase, preferably inhibiting the expression or the activity of the endogenous erythritol dehydrogenase of sequence SEQ ID NO: 7 (YALI_EYD1) or of an endogenous erythritol dehydrogenase having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the sequence SEQ ID NO: 7 (YALI_EYD1). Also for this aspect of the invention, said method may optionally further comprise overexpressing at least one enzyme selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and invertase as defined above, preferably a glycerol kinase and/or a transketolase. Preferably said method comprises overexpressing the glycerol kinase encoded by the *GUT1* gene and/or the transketolase encoded by the *TKL1* gene.

The present invention also provides a mutant erythritol and/or erythrulose-producing yeast strain, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous L-erythrulose kinase as defined above is inhibited and optionally wherein at least one enzyme selected from the group consisting of a erythritol dehydrogenase, a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase as defined above, preferably a glycerol kinase or a transketolase, is overexpressed, and more preferably a glycerol kinase and a transketolase are overexpressed.

The present invention also provides a mutant erythritol-producing yeast strain, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the

expression or the activity of the endogenous L-erythrulose kinase as defined above is inhibited and optionally at least 1, 2, 3, 4, 5, 6 or the 7 enzymes selected from the group consisting of glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase as defined above, preferably at least a glycerol kinase or a transketolase are overexpressed.

More preferably, a glycerol kinase and a transketolase as defined above are overexpressed in the mutant erythritol-producing yeast strain, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous L-erythrulose kinase as defined above is inhibited. More advantageously in this mutant, the glycerol kinase is encoded by the *GUT1* gene and the transketolase is encoded by the *TKL1* gene.

The present invention also provides a mutant erythrulose-producing yeast strain, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous L-erythrulose kinase as defined above is inhibited and an erythritol dehydrogenase as defined above is overexpressed and optionally at least 1, 2, 3, 4, 5, 6 or the 7 enzymes selected from the group consisting of glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase as defined above, preferably at least a glycerol kinase or a transketolase, is overexpressed.

Even more preferably, a glycerol kinase and a transketolase as defined above are overexpressed in addition to the erythritol dehydrogenase as defined above, in the mutant erythrulose-producing yeast strain, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous L-erythrulose kinase as defined above is inhibited. More advantageously in this mutant, the glycerol kinase is encoded by the *GUT1* gene, the transketolase is encoded by the *TKL1* gene and the erythritol dehydrogenase is encoded by the *EYD1* gene.

The present invention also provides a mutant erythritol-producing yeast strain without production of erythrulose, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous L-erythrulose kinase as defined above is inhibited and the expression or the activity of the endogenous erythritol dehydrogenase as defined above is inhibited and optionally at least 1, 2, 3, 4,

5, 6 or the 7 enzymes selected from the group consisting of glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase as defined above, preferably at least a glycerol kinase or a transketolase, is overexpressed.

Even more preferably, a glycerol kinase and a transketolase as defined above are overexpressed, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous L-erythrulose kinase as defined above and of the endogenous erythritol dehydrogenase as defined above is inhibited. More advantageously in this mutant, the glycerol kinase is encoded by the *GUT1* gene and the transketolase is encoded by the *TKL1* gene.

The present invention also provides a mutant erythritol-producing yeast strain without production of erythrulose, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous erythritol dehydrogenase (EC 1.1.1.9) as defined above is inhibited and optionally wherein at least one enzyme selected from the group consisting of a glycerol kinase (EC 2.7.1.30), a glycerol-3P dehydrogenase (EC 1.1.5.3), a triose isomerase (EC 5.3.1.1), a transketolase (EC 2.2.1.1), an erythrose 4 phosphate phosphatase (EC 3.1.3.23), an erythrose reductase (EC 1.1.1.21) and an invertase (EC 3.2.1.26), preferably a glycerol kinase and/or a transketolase, is overexpressed in said strain.

Said mutant yeast strain can be obtained by the method for obtaining a mutant erythritol and/or erythrulose-producing yeast strain as described above.

The mutant yeast strain of the invention includes not only the yeast cell resulting from the initial mutagenesis or transgenesis, but also their descendants, as far as the expression or the activity of the endogenous L-erythrulose kinase is inhibited and optionally as far as at least one enzyme selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase and an invertase as defined above, preferably a glycerol kinase and/or a transketolase, is overexpressed.

The present invention also provides a mutant erythritol and/or erythrulose-producing yeast strain, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous L-erythrulose kinase as defined above is inhibited and optionally further comprising, stably integrated in its

genome, at least one recombinant DNA constructs for expressing at least one enzyme selected from the group consisting of a glycerol kinase, a glycerol-3P dehydrogenase, a triose isomerase, a transketolase, an erythrose 4 phosphate phosphatase, an erythrose reductase, an invertase and an erythritol dehydrogenase as defined above, preferably a glycerol kinase and/or a transketolase and/or an erythritol dehydrogenase as defined above, and even more preferably the glycerol kinase encoded by the *GUT1* gene and/or the transketolase encoded by the *TKL1* gene and/or an erythritol dehydrogenase encoded by the *EYD1* gene.

Similar embodiments relating to a mutant erythritol-producing yeast strain without production of erythrulose, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous L-erythrulose kinase as defined above is inhibited, wherein the expression or the activity of the endogenous erythritol dehydrogenase as defined above is inhibited and optionally comprising, stably integrated in its genome, at least one recombinant DNA constructs for expressing at least one enzyme as defined above, are also provided by the present invention. As well as those relating to a mutant erythritol-producing yeast strain without production of erythrulose, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of the endogenous erythritol dehydrogenase as defined above is inhibited and optionally comprising, stably integrated in its genome, at least one recombinant DNA constructs for expressing at least one enzyme as defined above.

The present invention also provides the use of a mutant yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, of the invention for producing erythritol and/or erythrulose.

The present invention also provides the use of a mutant yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, of the invention for bioconverting erythritol to erythrulose.

The method for enhancing the erythrulose productivity and/or yield of an erythrulose-producing yeast strain according to the present invention can further comprise a step of culturing said erythrulose-producing yeast strain at a biomass comprised between 1 g and 150 g CDW/L, preferably between 10 g and 50 g CDW/L,

in a medium comprising an erythritol concentration comprised between 1 g/L and 200 g/L, preferably between 10 g/L and 80 g/L.

The present invention also provides a method for producing erythritol and/or erythrulose, comprising a step of growing a mutant yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, of the invention.

The present invention also provides a method for producing erythrulose or bioconverting erythritol to erythrulose, comprising a step of growing a mutant yeast strain, preferably a mutant *Yarrowia* strain, more preferably a mutant *Y. lipolytica* strain, of the invention, at a biomass comprised between 1 g and 150 g CDW/l, preferably between 10 g and 50 g CDW/l, in a medium comprising an erythritol concentration comprised between preferably between 1 g/L and 200 g/L, more preferably between 10 g/L and 80g/L.

Methods for extracting and purifying erythritol produced by cultured yeast strains are well known to those skilled in the art, *e.g.*, patent application EP 0 845 538; Rymowicz *et al.*, 2008; Moon *et al.*, 2010; Tomaszewska *et al.*, 2012; Mirończuk *et al.*, 2014.

Method for purifying erythrulose is described in Morii *et al.* (1985). HPLC method for erythrulose quantification is described in Ge *et al.* (2012).

NMR method for identifying erythritol and erythrulose are described in Nishimura *et al.*, (2006) and Hirata *et al.* (1999).

The mutant yeast of the invention can be cultured in repeated batch, fed-batch on continuous cultures as planktonic cell or biofilm (*i.e.*, cell growing on the surface or inside a solid support).

Advantageously, the source of carbon can be glycerol, glucose, sucrose, xylose, molasses, preferably glycerol.

The present invention will be understood more clearly from the further description which follows, which refers to non-limitative examples illustrating the inhibition of the expression of the YALI0F01606g gene encoding EYK1 of SEQ ID NO: 1 in *Y. lipolytica*, as well as to the appended.

Figure 1. Panel A shows the growth curve of *Y. lipolytica* strain W29 (□; empty square) JMY4949 (●; filled circle) and FCY001 (▲; filled triangle) during shake-flask culture in minimal YNBG and YNBE medium. Panel B shows the growth curve of *Y.*

lipolytica strain W29 on medium YNBG (○; empty circle), RIY208 on medium YNBG (△; open triangle) and RIY208 on medium YNBE (▲; filled triangle). Cultures were performed in shake flask.

Figure 2 shows the schematic representation of the insertion locus of the mutagenesis cassette (MTC, grey) in the YALI0F01606 gene (black) in the JMY4949 genome. Primers are indicated by the small arrow.

Figure 3 shows the glycerol and erythritol concentration in the culture medium (A) and cell growth (B) during shake-flask culture of erythritol production from W29 and FCY001. Panel A : ○ (empty circle): glycerol (W29); Δ (empty triangle): glycerol (FCY001); ● (filled circle): erythritol (W29); ▲ (filled triangle): erythritol (FCY001). Panel B: ○ (empty circle): glycerol (W29); Δ (empty triangle): glycerol (FCY001); ● (filled circle): biomass W29; ▲ (filled triangle): biomass FCY001.

Figure 4 shows the CLUSTAL multiple sequence alignment of *EYK1* genes in the *Yarrowia* clade performed by MUSCLE (3.8). Sequences are from strains YALI: *Yarrowia lipolytica* CLIB122 (100%); YAGA: *Yarrowia galli* CBS 9722 (96.77%); YAYA: *Yarrowia yakushimensis* CBS 10253 (91.62%); YAAL: *Yarrowia alimentaria* CBS 10151 (87.22%) and YAPH: *Yarrowia phangnensis* CBS 10407 (85.01%). Maximal identities with *Yarrowia lipolytica* EYK1 are indicated in brackets.

Figure 5 shows the HPLC analysis of culture supernatant of strains FCY001 and JMY2900 grown in YNBcasa containing 10 g/l of erythritol (ERY) or glucose (GLU). Chromatograms correspond to the U.V. signal recorded at 210 nm between 9 and 10 min of analysis. Samples were analysed in the presence (+) or in absence (-) of polyol standards at a final concentration of 2 g/L.

Figure 6 shows NMR spectra of culture supernatants of strain W29 and FCY001. A: Erythrulose solution at 2 g/L in D_2O. B: Culture supernatants of the *Y. lipolytica* wild-type strain W29. C: Culture supernatants of strain FCY001.

Figure 7 shows erythritol production (plain line) and glycerol consumption (doted line) for FCY218 (GUT1-TKL1-Δeyk, triangle) and JMY2900 (WT, circle) during culture in bioreactor in EPB medium.

Figure 8 shows relative expression of the genes *GUT1* and *TKL1* in strain FCY205, FCY208 and FCY214. The expression levels were standardized relative to the expression of the actin gene (ΔC_T); then the fold difference was calculated ($2^{-\Delta\Delta CT}$) based on baseline expression in the wild type strain W29.

EXAMPLES:

1) Material and methods

1.1) *Strains and media*

Wild-type *Y. lipolytica* strains used in this study are:

- W29 (*MATa ; Ery+*) (Barth and Gaillardin, 1996)
- Po1d *(MATa ura3-302, leu2-270 xpr2-322 ; Ura-, Leu-, Ery+*) (Barth, Gaillardin, 1996)
- JMY2900, prototrophe derivative of Po1d used as WT control, (*MATa ura3-302, leu2-270 xpr2-322 ; Ura+, Leu+, Ery+ ;*Po1d, Ura+, Leu+) (Ledesma-Amaro et al., 2015)
- JMY2101 (Leu+ derivative of Po1d, *MATa ura3-302, xpr2-322 ;Ura-,* Leu+, Ery+) (Leplat *et al.*, 2015)
- JMY4174 (*MATa ura3-302 leu2-270 xpr2-322* Δ*dga1*, Δ*lro1*, Δ*pox1-6*, *LEU2;*Ura-Leu+, Ery+)

Standard YPD and YNB media used for growth and transformation of *Y. lipolytica* were as described elsewhere (Fickers *et al.*, 2003). YNBG and YNBE used for mutant screening consisted of YNB medium with glucose replaced respectively by 1% (w/v) glycerol or 1% (w/v) erythritol. For erythritol production, media used were based on Tomaszewska *et al.* (2012). Growth medium (EG) consisted of (per liter): glycerol 50 g; peptone 5 g; yeast extract 5 g. Production medium used for shake-flasks cultures (EPF) was (per liter): glycerol 100 g; yeast extract 1 g; NH_4Cl 4.5g; $CuSO_4$ 0.7 x 10^{-3} g; $MnSO_4$. H_2O 32 x 10^{-3} g; 0.72 M phosphate buffer at pH 4.3. Production medium for bioreactor production (EPB) was (per liter): glycerol 150 g; NH_4Cl 2 g; KH_2PO_4 0.2 g; MgSO4 x 7 H_2O 1 g; yeast extract 1 g; NaCl 25 g.

Other *Y. lipolytica* strains used herein are the following:

- JMY4949 (JMY4174 derivative, YALI0F01606::MTC-*URA3*); *MATa ura3-302 leu2-270 xpr2-322* Δ*dga1*, Δ*lro1*, Δ*pox1-6*, *LEU2* YALI0F01606::MTC-*URA3;*Ura+ Leu+, Ery-) ;
- FCY001 (JMY2101 derivative, YALI0F01606::MTC-*URA3*), *MATa ura3-302, xpr2-322* YALI0F01606::MTC-*URA3;*Ura+, Leu+, Ery-;

- RIY208 (JMY2101 derivative, Δ*eyk1::URA3), MATa ura3-302, xpr2-322* Δ*eyk1*::*URA3;*Ura+, Leu+, Ery-;
- RIY203 (Po1d, Δ*eyk), MATa ura3-302 leu2-270 xpr2-322* Δ*eyk1;* Ura-, Leu-, Ery-;
- FCY205 (Po1d, *LEU2ex*-pTEF-*GUT1*, *URA3ex)*, *MATa ura3-302 leu2-270 xpr2-322 LEU2ex*-pTEF-*GUT1*, *URA3ex,* Ura+, Leu+, Ery+;
- FCY208 (Po1d, *URA3ex*-pTEF-*TKL1*, *LEU2)*, *MATa ura3-302 leu2-270 xpr2-322 URA3ex*-pTEF- *TKL1*, *LEU2,* Ura+, Leu+, Ery+
- FCY214 (Po1d, *LEU2ex*-pTEF-*GUT1*, *URA3ex*-pTEF-*TKL1)*, *MATa ura3-302 leu2-270 xpr2-322 LEU2ex*-pTEF-*GUT1 URA3ex*-pTEF- *TKL1*, *URA3ex,* Ura+, Leu+, Ery+
- FCY218 (Po1d, Δ*eyk, LEU2ex*-pTEF-*GUT1*, *URA3ex*-pTEF-*TKL1)*, *MATa ura3-302 leu2-270 xpr2-322 LEU2ex*-pTEF-*GUT1 URA3ex*-pTEF- *TKL1*, Ura+, Leu+, Ery-
- RIY146 *MATa ura3-302 leu2-270 xpr2-322* Δ*eyk1::LEU2*
- RIY210 (RIY145, *LEU2*), *MATa ura3-302 leu2-270 xpr2-322* Δ*eyk1::LEU2 URA3*ex-pTEF-*EYK1;* Ura+, Leu+, Ery-;

1.2) Culture conditions

All shake-flask cultures were performed at 28°C in 250 mL flasks containing 50 mL of appropriate medium. Shake-flasks mutant screening cultures were carried in YNBE or YNBG for 11 h at 190 RPM after a 24 h YPD growth. Erythritol productions were carried in EPF medium for 10 days at 250 RPM after a 72 h EG growth. All cultures were performed in triplicates.

Bioreactors cultures were performed in 2-l bioreactors (Biostat B-Twin, Sartorius) containing 1 L EPB medium at 28°C for 96 h, after a 72 h EG growth. Stirrer speed was set at 800 RPM and aeration rate was kept at 1 $vvmin^{-1}$. pH was set at 3.0 and automatically adjusted by the addition of 20% (w/v) NaOH or 40% (w/v) H_3PO_4. Bioreactor cultures were performed in duplicates.

1.3) Analytical methods

Cell growth was monitored by optical density at 600 nm (OD600) and dry cell weight (DCW) was calculated either from OD600 according to gDCW=OD600nm/4.7 or based on the biomass according to gDCW=OD600nm*0,29. Glycerol, erythritol and erythrulose concentrations in the media were determined by isocratic UV-RID-HPLC (Agilent 1100 series, Agilent Technologies) using an Aminex HPX-87H ion-exclusion column (300×7.8 mm Bio-Rad, Hercules, USA) with 15 mM Trifluoroacetic acid as mobile phase at a flow rate of 0.6 $ml.min^{-1}$ at 65 °C. Samples were analyzed using refractive index and absorbance at a wavelength of 205 nm. Compounds were identified on the basis of the retention time using commercially available standards. Glycerol concentration was calculated from HPLC chromatogram based on the following calibration equations: glycerol concentration = [(pic area -1888)/66307] or glycerol concentration = [(pic area -1879)/76916].

1.4) General molecular biology techniques

Standard molecular biology techniques were used (Green *et al.*, 2012). Transformation and genetic manipulations of *Y. lipolytica* were done according to Barth and Gaillardin (1996). Genomic DNA from *Y. lipolytica* was prepared according to Querol *et al.*, (1992). PCR reactions were performed on a MJ Mini Gradient Thermal Cycler (Bio-Rad) using DreamTaq DNA polymerase (Thermo Scientific), except for genome walking PCR (see below). 25 cycles were carried for each PCR reaction, and were as follows: denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min/kb. A final 10 min extension was added as the last step. PCR fragments were purified from agarose gels using GeneJet Gel Extraction Kit (Thermo Scientific).

1.5) Mutant library screening

A library of randomly generated *Y. lipolytica* mutants was constructed by inserting a mutagenesis cassette (MTC) in the genome of the *Y. lipolytica* wild-type strain JMY4174 (Ura-). The MTC sequence consisted of two zeta regions from Ylt1 retrotransposon, allowing random genome insertion (Barth and Gaillardin 1996), flanking the URA3 gene for selection. 11,000 mutants were obtained and screened at the PICT-Genotoul Platform (INSA-Toulouse). After two growth phases on liquid YNB

with 2% and 0.2% glucose concentrations respectively, the mutants were screened on two different solid media, YNBG and YNBE.

Colonies exhibiting normal growth on glycerol but slow growth on erythritol were selected for a second screening. After further growth on YNB, two replicates of each selected mutant were transferred on new plates containing YNBG or YNBE. The clones still showing a slow growth on erythritol for both replicates were selected for shake-flask screening, as described above.

1.6) Genome Walking

The insertion site of the MTC in JMY4949 strain was identified by genome walking using Universal GenomeWalker 2.0 (ClonTech Laboratories inc.). After extraction, genomic DNA was digested with four different restriction enzymes (DraI, EcoRV, PvuII, StuI) and the resulting fragments were ligated with the GenomeWalker adaptors. PCR reactions were performed on the ligated fragments using primers matching the adaptor (AP1, see Table 1) and either the 5' side (GSP1-L) or the 3' side (GSP1-R) of the MTC. This allowed to amplify only the genomic fragments containing the MTC and its surroundings.

A second PCR reaction with different primers (AP2 and either GSP1-L or GSP1-R) was then performed to ensure specificity. The PCR steps were performed using Advantage 2 Polymerase (ClonTech Laboratories inc.) and cycles were designed as recommended by the user manual. The resulting amplified fragments were separated by gel electrophoresis, purified, and sequenced with Sanger sequencing (GATC Biotech). A BLAST analysis of the sequences was then performed at the GREC site (http://gryc.inra.fr/) on the *Y. lipolytica* genome to identify the insertion site of the MTC.

1.7) Disruption of YALI0F1606g in a wild-type strain

Construction of the FCY001 strain was achieved by disrupting the YALI0F01606g gene within JMY2101 strain. A 3700 base pairs (bp) region consisting of the MTC insertion site and its surroundings (1000 bp on each side of the MTC insertion site) was amplified from JMY4949 strain, using primers DISR1 and DISR2. The amplified fragment was analyzed by gel electrophoresis and purified. This fragment contained all the elements for a disruption cassette of YALI0F01606g; specific

sequences for homologuous recombination and site-directed insertion, and a selection marker (*URA3* gene within the MTC). This purified disruption cassette was used to transform JMY2101 strain. Transformed strains were selected on YNB plates, and the success of the gene disruption was verified by PCR, using ZETA1 and CHK1 primers.

Strain RIY208 was constructed by disrupting the *EYK1* gene in strain JMY2101 as described hereinafter. The *EYK1* P and T fragments were amplified from strain W29 genomic DNA using primer pairs EYK1-PF/EYK1-PR and EYK1-TF/EYK1-TR, respectively. The *URA3* marker was amplified from the JMP113 plasmid (Fickers et al. 2013) using the primer pair LPR-F/LPR-R. Primer EYK1-PR, EYK1-TR, LPR-F and LPR-R were designed to introduce an *Sfi*I restriction site in amplified fragment. Amplicons were digested with *Sfi*I before being purified and ligated, using T4 DNA ligase, at a molar ratio of 1:1. The ligation products were amplified via PCR using the primer pair EYK1-PF/EYK1-TR. They were then purified and used to transform strain JMY2101, this process yielded strain RIY208 (Δ *eyk1::URA3)*. The prototroph derivative of strain RIY208, namely RIY203 was obtained according to Fickers et al. 2003.

Strain RIY203 was constructed using the same disruption cassette except that the transformed strain was Po1d. This process yielded strain RIY203.

1.8) Strain construction for overexpression of glycerol kinase and transketolase

The different genes that were over-expressed are YALI0F00484g *(GUT*1, Glycerol kinase, *Y. lipolytica; BamHI* site removal) and YALI0E06479g *(TKL1,* Transketolase *Y. lipolytica;* Intron removal, *ClaI* site removal). Yeast genes were amplified from genomic DNA of strain *Y. lipolytica* W29.

Primers for gene amplification were designed to introduce an *Avr*II site at the 3' end and a *Bam*HI restriction sites at the 5' end of genes YALI0F00484g and YALI0E06479g (Table 1). Introns and undesirable restriction sites were removed by overlap extension PCR and site-directed mutagenesis (Higuchi et al., 1988): *BamHI* site removal in YALI0F00484g (*GUT*1, Glycerol kinase, *Y. lipolytica*) was performed with primer GUT1F1/GUT1R1 (PCR1) and GUT1F2/ GUT1F1 (PCR2) and finally with

GUT1F1/ GUT1F1 using amplicons from PCR1 and PCR2 as templates. Intron removal, *ClaI* site removal for YALI0E06479g *(TKL1,* Transketolase *Y. lipolytica*.) was performed using primer pairs *TKL1F1/TKL1R1* (PCR1), *TKL1F2/*TKL1R2 (PCR2) and *TKL1F3/TKL1R3*, (PCR3). Finally, the modified *TKL1* was amplified with primers TKLF1/TKL1R3 and amplicons from PCR1, PCR2 and PCR3 as template.

Amplicons were purified from agarose gel, before being digested using *BamH*I/*Avr*II restriction enzymes. The corresponding fragments were finally cloned into *BamH*I/*Avr*II digested JMP1047 (Lazar et al 2013) or JMP2563 (Dulermo et al 2017) vectors in order to obtain *URA3* or *LEU2* counterpart, respectively. The correctness of the resulting construct was verified by DNA sequencing.

Expression cassettes for genes *GUT1* and *TKLI* were rescued from corresponding vectors by *Not*I digestion and purified from agarose gel before being used to transform *Y. lipolytica* strains Po1d or RIY203. Transformants were selected on YNB medium supplemented with uracil or leucine depending on their auxotrophy. Correctness of the constructed strain was verified by analytical PCR on genomic DNA using primer pairs URA3F/61stop or LEU2F/61stop, depending on the auxotrophic marker used for transformation. Prototrophic stains were obtained according to Fickers et al. 2003.

Table 1: Primers used for genome walking and strain constructions (Restriction sites are underlined, mismatched bases for site-directed mutagenesis are in bold, overhangs for overlap extension PCR are in italics) are the following:

Primer	Sequence (5'- 3')	SEQ ID No.
GSP1-L	TCTCGGTGGTCAATGCGTCAGAAGATATC	13
GSP2-L	AGCCGAGTGAATGTTGCCTGCCGTTAGT	14
GSP1-R	AGCGTTCGCCAATTGCTGCGCCATCGT	15
GSP2-R	ACACTACCGAGGTTACTAGAGTTGGGAAA	16
AP1	GTAATACGACTCACTATAGGGC	17
AP2	ACTATAGGGCACGCGTGGT	18
DISR1	TGTAGCACCTGGGTCAACATTT	19
DISR2	TCCGATGACCTGACTAGTGCG	20
CHK1	GATTGCTCCGTTTGTAAGTACA	21
ZETA1	TGGTCCTGTTCCACCTGAAC	22

Primer	Sequence (5'- 3')	SEQ ID No.
GUT1 F1	GACGGATCCATGTCTTCCTACGTAGGAGCTCTC (restriction site *Bam*HI)	23
GUT1 R1	GTTATCCAGAATCCATCGGAC	24
GUT1 F2	GGTCCGATGGATTCTGGATA	25
GUT1 R2	GACCCTAGGTTACTCAAGCCAGCCAACAG (restriction site *Avr*II)	26
TKL1 F1	CGAGGATCCATGGCTCCCCAATTTTCAAAG (restriction site *Bam*HI)	27
TKL1 R1	GCCACAGCATCAATG*CCAAGGTTCGGATGGTGTT*	28
TKL1 F2	*ATCAACACCATCCGAACCTTGGCT*ATTGATGCTGTGGCCAAGGC	29
TKL1 R2	GTTCTTGAGATCATCAATAGTGATGTCGTAGC	30
TKL1 F3	GCTACGACATCACTATTGATGATCTCAAGAAC	31
TKL1 R3	GACCCTAGGTTAGACACCGTGGCCGGGTC (restriction site *Avr*II)	32
URA3 F	AGGAAGAAACCGTGCTTAAGAG	33
LEU2 F	TAAGTCGTTTCTACGACGCATT	34
61 Stop	GTAGATAGTTGAGGTAGAAGTTG	35
EYK-PF	GTTGTGTGATGAGACCTTGGTGC	36
EYK-PR	AAAGGCCATTTAGGCCGCAGCTCCTCCGACAATCTTG (restriction site SfiI)	37
EYK-TF	TAAGGCCTTGATGGCCACAAGTAGAGGGAGGAGAAGC (restriction site SfiI)	38
EYK-TR	GTTTAGGTGCCTGAAGACGGTG	39
LPR-F	ATAGGCCTAAATGGCCTGCATCGATCTAGGGATAACAGG (restriction site SfiI)	40
LPR-R	ATAGGCCATCAAGGCCGCTAGATAGAGTCGAGAATTACCCTG (restriction site SfiI)	41
GUT1-L-q	CCCTGTCCACCTACTTTGCC (target gene GUT1)	42
GUT1-R-q	TTGGAGGTGTCGGTGATGTG (target gene GUT1)	43
TKL1-P-L-q	CAGCAACACAGATGGCAACC (target gene GUT1 *TKL1)*	44
TKL1-T-R-q	CGAGACCTCCGCTGCTTACTAC (target gene GUT1 *TKL1)*	45
ACT-F	GGCCAGCCATATCGAGTCGCA (target gene *ACT)*	46
ACT-R	TCCAGGCCGTCCTCTCCC (target gene *ACT)*	47

1.9) RNA isolation and transcript quantification.

Shake-flask cultures were grown in EPF medium for 24h. Cells were then collected and store at -80°C. RNA extraction and cDNA synthesis were perfomed as

previously described (Sassi et al 2016). Primers for RT-qPCR are listed in Table 1. The results were normalized to actin gene and analyzed to the ddCT method (Sassi et al 2016). Samples were analyzed in duplicates.

2) Results

2.1) Mutant screening

In order to isolate a *Y. lipolytica* strain unable to grow on erythritol, a library of 11,000 insertion mutants was screened on glycerol and erythritol medium plates. After the first screening, 188 mutants were selected for having a have normal growth on glycerol but a slow growth on erythritol. After a second screening, 10 mutants were still displaying this phenotype consistently and were selected for shake-flask screening. Among these, one mutant was confirmed to be deficient for erythritol consumption (Figure 1A). No growth on erythritol was observed for this mutant, while it grew as fast as W29 on glycerol. This strain was named JMY4949.

2.2) Identification of the disrupted gene

In order to find which gene was disrupted in the JMY4949 strain, a genome walking analysis was performed. Primers designed to match the MTC allowed to amplify the region surrounding its insertion site in the JMY4949 genome. After sequencing this region, BLAST analysis revealed that the MTC insertion site was located within the YALI0F01606g gene, indicating that the disruption of this gene caused the loss of the ability to grow on erythritol (Figure 2).

2.3) Construction of a Y. lipolytica strain disrupted in YALI0F1606g gene

A disruption cassette of this gene YALI0F01606g was constructed to transform the wild-type strain JMY2101. The strain FCY001 was obtained as a result. This strain has the same genotype as W29 except for the disruption of YALI0F01606g. This strain was evaluated in shake-flasks in YNBG and YNBE medium, and exhibited the same phenotype as JMY4949 strain (Figure 1A). These results confirmed that the YALI0F01606g gene is essential in the erythritol catabolism pathway, and that the disruption of this gene alone is sufficient to remove the ability of *Y. lipolytica* to use erythritol as a carbon source. In addition, growth of FCY001 did not show any growth

defect on glycerol media (Figure 1A). As shown in Figure 1B, strain RIY208 which is also a strain disrupted in YALI0F1606g gene, shows a growth defect on YNBE medium. It showed a similar growth profile as compared to strain W29 on YNBG medium.

2.4) Shake-flask erythritol production

In order to assess the effects of a ΔYALI0F01606g strain on erythritol production, shake-flask production cultures were carried using W29 and FCY001 (Figure 3). After 7 days of culture and near glycerol exhaustion, FCY001 had produced 35.7 g/l erythritol while W29 had only produced 30.7 g/l, meaning that the disruption of YALIF01606g gene had a positive effect on erythritol production. Results also showed that as soon as glycerol was depleted, W29 strain began to use erythritol for its growth, leading to a quick decrease of erythritol concentration in the medium. On the other hand, only a small decrease in erythritol concentration was observed in the FCY001 culture, after which its concentration remained stable during at least seven days. The small drop in erythritol concentration might be due to a partial conversion of erythritol into L-erythrulose, which couldn't be further converted. This would be consistent with the hypothesis that YALI0F01606g is an EYK.

2.5) Bioreactor erythritol production

Batch bioreactor cultures of FCY001 and W29 were performed to further evaluate the benefits of a YALI0F01606g disruption in production conditions. Results are displayed in Table 2.

Table 2: Characteristic parameter of erythritol production during culture in bioreactor of W29 and FCY001

Parameters/ strain	FCY001	W29
Yield ($g.\ g^{-1}$)*	0.46 ± 0.15	0.34 ± 0.02
Yield ($g.\ g^{-1}$)$	0.49 ± 0.02	0.39 ± 0.01
Erythritol productivity ($g.\ l^{-1}.\ h^{-1}$)	0.59 ± 0.03	0,52 ± 0.05
Specific erythritol productivity ($g.\ l^{-1}.\ h^{-1}.\ DCW^{-1}$) ¤	0.115± 0.005	0,089 ± 0.002
Specific glycerol uptake rate ($g.\ l^{-1}.\ h^{-1}.\ DCW^{-1}$) ¤	0.291 ± 0.013	0.253 ± 0.005
Specific erythritol productivity ($g.\ g_{DCW}^{-1}.\ h^{-1}$) *	0.052± 0.005	0,040 ± 0.002
Specific glycerol uptake rate ($g.\ g_{DCW}^{-1}.\ h^{-1}$) *	0.110 ± 0.003	0.101 ± 0.003

* glycerol concentration was calculated according to glycerol concentration = [(pic area - 1888)/66307].

$ glycerol concentration was calculated according to glycerol concentration = [(pic area - 1879)/76916].

¤ specific productivity according to gDCW=OD600nm/4.7

* specific productivity according to gDCW=OD600nm*0,29

Bioreactor experiments confirmed the observations from the shake-flasks observations. Compared to W29, FCY001 had 25 to 35% higher yield depending on the method used for glycerol calculation, 28 to 30% higher specific productivity depending on the calculation method used for the conversion of the measured OD, and a 13% higher productivity. The significantly higher yield compared to the W29 strain might indicate that in a wild-type strain, some of the produced erythritol is consumed even before glycerol depletion. More surprising is the observation that FCY001 glycerol uptake is consistently faster than for W29, although its growth is slightly slower (data not shown), which would indicate that a YALI0F01606g disruption improves glycerol uptake, and that this increased glycerol uptake is mostly directed towards erythritol production rather than biomass production. These results altogether show that a YALI0F01606g disruption allows the improvement erythritol production while helping to keep its concentration stable after glycerol depletion.

2.6) Shake-flask erythrulose production

In order to further assess the effects of the disruption of YALI0F01606g on *Y. lipolytica* phenotype, strain FCY001 and JMY2900 were grown in YNBCasa medium supplemented with glucose or erythritol. Cultures were inoculated at a relatively high biomass (*i.e.*, 0.5 g CDW/ml) and medium was supplemented with casamino acid as energy source for strain FCY001 since this latter has been demonstrated to be unable to grow on YNB-erythritol (Figure 1A). After 48 h of culture at 28 °C, biomasses were equal to 1 and 4 g CDW/ml for strain FCY001 and JMY2900, respectively. Culture supernatants were analyzed by HPLC for the presence of erythritol or erythrulose. For strain JMY2900, erythritol was not detected whereas a residual concentration of 2.6 g/L was measured in culture supernatant of strain FCY001 (data not shown). Figure 5 shows the UV signals recorded for culture supernatant, pure or mixed with erythrulose or

erythritol. For strain FCY001 supernatant, two compounds were eluted at retention time 9.186 and 9.658 min. Based on the chromatogram obtained for supernatants of strains FCY001 and JMY2900 grown on erythrulose and glucose based medium, respectively, these two compounds seems to be related to erythritol catabolism and to be specific of FCY001 mutant. Moreover, addition of pure erythritol in the sample did not modify the elution profile demonstrating that these two compounds do not correspond to erythritol. By contrast, addition of pure erythrulose in the sample, led to an increase of the elution peak intensity of one of the two compounds demonstrating, thus, that it corresponds to erythrulose.

The defect of growth observed for FCY001 in the presence of erythritol together with the detection of erythulose in the culture supernatant of this strain demonstrate clearly that gene YALI0F01606g is involved in erythitol catabolism and that it corresponds to erythritol kinase.

2.7) Erythrulose production analysis by NMR

To confirm that the disruption of *EYK1* lead to the accumulation of erythrulose, strains FCY001 and wild-type strain W29 were incubated at high cell density in EPF medium for 48 h and, the culture supernatants were analyzed by NMR spectroscopy. For that purpose, EPF medium was inoculated at high cell density (OD 600 nm = 2) with *Y. lipolytica* strains and incubated for 48 h at 250 RPM. Culture supernatants were then used for NMR measurements. Spectra were recorded at 25°C on a Bruker AVIII HD equipped with a SMART BBFO probe operating at 400MHz for the ^{1}H. The pulse sequence used for ^{1}H detection with water suppression was Perfect-echo Watergate sequence (Adams et al 2013). Spectra were centered on the water signal at 4.7 ppm. 16 transient were added on 32K point during an acquisition time of 2.56 s. The delay for binomial water suppression was 800 µs and the relaxation delay was 1 s. Prior to Fourier transform, data were multiplied with an exponential function to give a broadening of 0.3Hz. Samples were prepared by mixing 570 µl of *Y. lipolytica* culture supernatant with 30 µl of D_2O. Erythrulose (Sigma Aldrich) solution at 2 g/L in D_2O was used as a standard.

As shown in figure 6, the characteristic signals observed for erythrulose standard solution in the range of 4.32 and 4.54 ppm are clearly present for strain FCY001 as

compared to strain W29. This clearly demonstrated that the *EYK* disrupted strain accumulates erythrulose as compared to the non-disrupted strain.

2.8) The pull and push strategy to enhance erythritol production

Overexpression of glycerol kinase increase glycerol assimilation rate and erythritol productivity

For strain FCY205 (pTEF-*GUT1*), the specific glycerol consumption rate (q_{GLY}) was increased by 20 % as compared to the parental strain [i.e. 0.091 and 0.076 g/(gDCW h), respectively] (Table 3). This increase is in the same range as that obtained for *Y. lipolytica* strain A101 overexpressing *GUT1* (Mironczuk et al 2016).

In strain overexpressing *GUT1* (FCY205), erythritol specific productivity (q_{ERY}) was increased by 45 % as compared to the wild-type strain [i.e. 0.051 and 0.035 g/(gDCW h), respectively] while yield was increased by a 21 % [i.e. 0.56 and 0.46 g/g, respectively].

Overexpression of triose isomerase and transketolase leads to an increase in erythritol productivity

Gene encoding *TKL1* involved in erythritol synthesis from DHAP, the end product of glycerol catabolism, identified in *Y. lipolytica* genome as YALI0E06479g, was used to construct strains FCY208.

Strain FCY208 (p*TEF-TKL1*) also showed a higher conversion yield ($Y_{S/P}$) as compared to FCY205 (p*TEF-GUT1*) [i.e. 0.59 and 0.56 g/g, respectively; Table 3]. However, glycerol uptake was found somewhat lower for this mutant (0.068 $g.g_{DCW}^{-1}.h^{-1}$) as compared to the wild-type strain (0.076 $g.g_{DCW}^{-1}.h^{-1}$).

Strain FCY205 (p*TEF-GUT1*) has shown a significant increase in glycerol uptake capacity while strain FCY208 (p*TEF-TKL1*) was able to convert glycerol into erythritol with the highest yield. To further increase erythritol productivity, these two genes were co-expressed in strain FCY214. In shake flask culture, this strain performed significantly better than JMY2900 in term of erythritol specific productivity (i.e. 65% increase) and cumulates the positive effect observed for strains FCY205 and FCY208,

i.e. higher glycerol uptake rate [i.e.0.095 and 0.091 g/L, respectively] and higher glycerol/erythritol conversion yield [i.e. 0.61 and 0.59 g/L, respectively].

Results are summarized in Table 3 below.

Table 3: Dynamic parameters calculated from glycerol uptake and erythritol synthesis after 8 days of culture in EPF medium for the different constructed strains

Strain	Overexpressed genes	Biomass (g_{DCW})	q_{ERY} ($g.g_{DCW}^{-1}.h^{-1}$)	q_{GLY} ($g.g_{DCW}^{-1}.h^{-1}$)	$Y_{S/P}$ ($g.g^{-1}$)
JMY2900 (WT)	-	5.30	0.035	0.076	0.46
FCY205	*GUT1*	4.83	0,051	0.091	0.56
FCY208	*TKL1*	5.36	0.040	0.068	0.59
FCY214	*GUT1-TKL1*	4.81	0.058	0.095	0.61

The values provided are the means of three independent replicates; the standard deviations were less than 10% of the mean. q_{ERY} erythritol specific production rate, q_{GLY} glycerol specific consumption rate, $Y_{S/P}$ glycerol/erythritol conversion yield.

Quantification of the overexpression of gene GUT1 and TKL1

Figure 8 shows that gene *GUT1* and *TKL1* are overexpressed in the corresponding strain (ie FCY205, FCY208 and FCY214) between 3 to 16 more than in strain JMY2900.

2.9) Overexpression of triose isomerase and transketolase in strain RIY203 further increases erythritol productivity

Overexpression of the genes *GUT1* and *TKL1* was carried out in a strain wherein the *EYK1* gene (YALI0F01606g) was disrupted.

Behavior of the resulting strain FCY218 and FCY214 were investigated in bioreactor as compared to strain JMY2900. Results are presented in Table 4 and Figure 7.

Table 4: Results of bioreactor cultures of FCY214 and FCY218. Standard deviation were less than 10%

	JMY2900	FCY214	FCY218
Erythritol ($g.l^{-1}$)	55.8	79.4	78.5
Productivity ($g.l^{-1}.h^{-1}$)	0.59	0.84	1.05
q_{ERY} ($g.g_{DCW}^{-1}.h^{-1}$)	0.046	0.057	0.071
q_{GLY} ($g.g_{DCW}^{-1}.h^{-1}$)	0.105	0.119	0.135
Yield ($g.g^{-1}$)	0.44	0.48	0.53
Final biomass (g_{DCW})	12.8	14.7	14.9

At the end of the culture of strain FCY214, erythritol concentration in the culture supernatant reached 79.4 $g.l^{-1}$. That is a significant increase (42 %) as compared to the parental strain (55.8 $g.l^{-1}$). In those conditions, erythritol is produced at a constant rate (0.84 g/L.h) between 24 and 96 h of culture (Table 4).

As expected, the resulting strain FCY218 is unable to reconsume erythritol, especially after glycerol exhaustion in the bioreactor (Figure 7). As a consequence, strain FCY218 showed a higher q_{GLY} as compared to FCY214 [i.e. 0.135 and 0.119 g/(gDCW h), respectively], a higher erythritol productivity [i.e. 1.05 and 0.84 g/L h^{-1}, respectively] and a higher yield [i.e. 0.53 and 0.48 g/g, respectively] (Table 4). Moreover, the maximal erythritol concentration was obtained in a lag of time reduced by 66 %, as compared to strain JMY2900, positively affecting the process profitability.

2.10) Overexpression of YALI0F01650g in a Δeyk strain allows the conversion of erythritol into erythrulose at high yield

Y. lipolytica gene YALI0F01650g (SEQ ID NO: 7) has 56% identity with gene ODQ69345.1 (SEQ ID NO: 48) and ODQ69163.1 (SEQ ID NO: 49) that encode erythritol dehydrogenase in *Lipomyces starkeyi*. From this YALI0F01650g was suggested to encode an erythritol dehydrogenase in *Y. lipolytica*. The disruption of the latter, renamed *EYD1*, impairs growth on erythritol medium.

Strain RIY210 was constructed by overexpressing YALI0F01650g under the strong constitutive promoter pTEF in strain RIY203. EYD was amplified from JMY2900 genomic DNA by PCR using primers EYD_Surexp_F (SEQ ID NO: 50 = GACGGATCCCACAATGGTTTCTTCAGCCGCTACTT) and EYD_surexp_R (SEQ ID NO: 51 = GACCCTAGGTTACCAGACGTGGTGGCCAC); designed to introduce a BamHI and AvrII restriction sites in the PCR fragment. The latter was cloned into BamHI/AvrII digested JMP1047 (Lazar et al 2013) vectors and used to transform strain RIY146. The resulting strain RIY210 was then grown in medium YNB containing a mixture of glycerol and erythritol (50/50). Accumulation of erythulose in culture supernatant was estimated by HPLC after 24 h of growth. Results were compared to that obtained for the wild-type strain. As shown in Table 5, erythrulose accumulate in the culture supernatant of strain RIY210. Conversion of erythritol into erythrulose is closed to 65%.

Table 5: accumulation of erythrulose in strain W29 and RIY210

	W29	**RIY210**
Biomass at t=0h (gDCW/L)	0.58	0.58
Biomass at t=24h (gDCW/L)	12.85	9.15
Glycerol consumed (g/L)	10	10
Erythritol consumed (g/l)	10.2	7.51
Erythrulose produced (g/L)	0	**4.83**
Yield (g/g)	0	**0.63**
Productivity (g/L.h)	0	**0.20**

Conclusions

The present invention provides mutant strains impaired in erythritol catabolism with erythritol productivity increased by 72% and a 65 % increase in erythritol specific productivity as compared to a wild-type strain, while process duration was reduced by 66 %. It also provides a mutant strain impaired in erythritol catabolism with a conversion of erythritol into erythrulose close to 65%. All these advantages were obtained using an inexpensive medium and in a non-optimized process.

REFERENCES

Altschul, S., *et al.*, 1997. *Nucleic Acids Res.* 25, 3389-3402.

Barth, G. and Gaillardin, C. 1996. *Yarrowia lipolytica*. In: *Nonconventional Yeasts in Biotechnology*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 313–388.

Barth, G. and Gaillardin, C. 1997. *FEMS microbiology reviews*, 19, 219–237.

Blazeck, J., 2011. *Applied and Environmental Microbiology*. 77: 7905–7914.

Blazeck, J., 2013. *Applied Microbiology and Biotechnology*. 97:3037-3052.

Blazeck J., *et al.*, 2014. *Nature Communications*. 5: 3131, DOI: 10.1038/ncomms4131.

Baykov, A.A., *et al.*, 1988. *Anal. Biochem.* 171, 266–270.

Carly F., *et al.*, 2015. Enhancement of erythritol production from *Yarrowia lipolytica* by metabolic engineering. In: *XVI Congreso Nacional de Biotecnología y Bioingeniería*. 21 al 26 de Junio de 2015, Guadalajara, Jalisco, México.

Dulermo R., Brunel F., Dulermo T., Ledesma‐Amaro R., Vion J., Trassaert M., Thomas S., Nicaud J-M. and Leplat C.. (2017) Using a vector pool containing variable‐strength promoters to optimize protein production in Yarrowia lipolytica. Microb Cell Fact (2017) 16:31. DOI 10.1186/s12934-017-0647-3

Fickers, P., *et al.*, 2003. *Journal of Microbiological Methods*, 55, 727–737.

Fickers, P., *et al.*, 2005. *FEMS Yeast Research*, 5, 527–543.

Gaillardin, C., Ribet, AM. 1987. *Current Genetics*. 11: 369-375.

Ge, C., *et al.*, 2012. *Se Pu*. 30, 843-846.

Green, M.R., *et al.*, 2012. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y, 3 pp.

Gysler, C., *et al.*, 1990, *Biotechnology Techniques*. 4, 285-290.

Hedfalk, K. 2012 'Codon Optimisation for Heterologous Gene Expression in Yeast'. In Springer Protocols :Methods in Molecular Biology. Recombinant protein production in yeast :methods and protocols. Volume 866 pp. 47-55. Springer Eds.

Hellingwerf, F.J., WO2015-147644A1.

Higuchi, R., Krummel, B., and Saiki, R.K. (1988). A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. *16*, 7351–7367.

Hirata, Y., *et al.*, 1999, *Journal of Bioscience and Bioengineering*, 87:630-635.

Ishizuka, H., *et al.*, 1989. *Journal of Fermentation and Bioengineering*, 68, 310–314.

Ishizuka, H., *et al.*, 1992. *Bioscience, Biotechnology, and Biochemistry* 56, 941-945.

Ito, H., *et al.*, 1983, *Journal of Bacteriology*. 153, 163-168.

Jeya, M., *et al.*, 2009. *Applied microbiology and biotechnology*, 83, 225–231.

Kim, S.-Y., *et al.*, 1997. *Biotechnology Letters*, 19, 727–729.

Klebe, R.J., *et al.*, 1983, *Gene*. 25:333-341.

Kuznetsova, E., *et al.*, 2006. *The Journal of Biological Chemistry*, 281, 36149–36161.

Lazar, Z. *et al.*, 2013, *J Ind Microbiol Biotechnol.*, 40, 1273–1283.

Lee, J.-K., *et al.*, 2000. *Biotechnology Letters*, 23, 497-500.

Lee, D., *et al.*, *Microbial Cell Factories*, 2010, 9, 43.

Le Dall, M.T., *et al.*, 1994, *Current Genetics*. 26:38-44.

Leplat, C., *et al.*, 2015, FEMS Yeast Research, doi: 10.1093/femsyr/fov052.

Lindgren, V., *et al.*, 1977, *Journal of Bacteriology*, 119, 431-442.

Madzak, C., *et al.*, 2000, *Journal of Molecular Microbiology and Biotechnology*, 2:207-216.

Madzak, C., *et al.*, 2004, *Journal of Biotechnology*,109:63-81.

Maftahi, M., *et al.*, *Yeast*, 1996, 12:859-868.

Matsushika, A., *et al.*, 2012, *Enzyme and Microbial Technology*, 51, 16-25.

Miller, G.L., 1959, *Anal Chem* 31, 426–428.

Mirończuk, A.A., *et al.*, 2014, *Journal of Industrial Microbiology and Biotechnology*, 41:57-64.

Mizanur R.M., *et al.*, 2001, *J. Biosc. Bioeng.*, 92:237-241.

Moon, H.-J., *et al.*, 2010. *Applied Microbiology and Biotechnology*, 86, 1017–1025.

Moonmangnee, D., *et al.*, 2002, *Biosci. Biotechnol. Biochem.*, 66:307-318.

Morii, K., *et al.*, 1985, *Anal Biochem.*, 151, 188-191

Müller, S., *et al.*, 1998. *Yeast*. 14:1267–1283.

Nicaud, J-M, *et al.*, 2002. *FEMS Yeast Research* 2/3, 371-379.

Nicaud, J.-M., 2012. *Yeast*, 29, 409–418.

Nishimura, K., *et al.*, 2006. *Journal of Bioscience and Bioengineering*; 101:303-308.

Orr-Weaver, T.L., *et al.*, 1981, *Proceedings of the National Academy of Sciences of the United States of America*, 78:6354-6358.

Querol, A., *et al.*, 1992. *Applied and Environmental Microbiology*, 58, 2948–2953.

Paradowska and Dagmara (2009). *Ann. Universatis Marie-Curie Sklodowska Lublin*, 13 :47-54.

Ratledge, C. (1994). Yeasts, moulds, algae and bacteria as sources of lipids. Technological advances in improved and alternative sources of lipids. B. S. Kamel, Kakuda, Y. London, Blackie academic and professional, 235-291.

Rymowicz, W., *et al.*, 2008. *Biotechnology Letters*, 31, 377–380.

Ryu, Y.-W., *et al.*, 2000. *Journal of Industrial Microbiology and Biotechnology*, 25, 100–103.

Rywińska, A., *et al.*, 2013. *Biomass and Bioenergy*, 48, 148–166.

Sassi, H., et al 2016, *Microbial Cell Factories*, 15 :159

Song, P., *et al.*, 2011. *Fungal Biology*, 115, 49–53.

Sprague, G. F., *et al.*, 1977. *Journal of Bacteriology*, 129, 1335-1342.

Sharma, S., *et al.*, 2012. *Plant Signaling and Behaviour*, 7, 1337-1345.

Tomaszewska, L., *et al.*, 2012. *Journal of Industrial Microbiology & Biotechnology*, 39, 1333–1343.

Wang, H.J., *et al.*, 1999, *Journal of Biotechnology*, 181:5140-5148

Wu, Z.L., 2011, *PLoS One*, 6, e23172.

Zinjarde, S.S., 2014. *Food chemistry*, 152, 1–10.

CLAIMS

1. A method for enhancing the erythritol or erythrulose productivity and/or yield of an erythritol and/or erythrulose-producing yeast strain, characterized in that it comprises inhibiting in said yeast strain the expression or the activity of an endogenous L-erythrulose kinase (EC 2.7.1.27) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 1 (YALI_EYK1).

2. The method for enhancing the erythritol productivity and/or yield of an erythritol-producing yeast strain according to claim 1, characterized in that it further comprises overexpressing in said strain at least one enzyme selected from the group consisting of a glycerol kinase (EC 2.7.1.30), a glycerol-3P dehydrogenase (EC 1.1.5.3), a triose isomerase (EC 5.3.1.1), a transketolase (EC 2.2.1.1), an erythrose 4 phosphate phosphatase (EC 3.1.3.23), an erythrose reductase (EC 1.1.1.21), and an invertase (EC 3.2.1.26), preferably a glycerol kinase and/or a transketolase.

3. The method for enhancing the erythrulose productivity and/or yield of an erythrulose-producing yeast strain according to claim 1, characterized in that it further comprises overexpressing in said strain an erythritol dehydrogenase (EC 1.1.1.9) and optionally at least one enzyme selected from the group consisting of a glycerol kinase (EC 2.7.1.30), a glycerol-3P dehydrogenase (EC 1.1.5.3), a triose isomerase (EC 5.3.1.1), a transketolase (EC 2.2.1.1), an erythrose 4 phosphate phosphatase (EC 3.1.3.23), an erythrose reductase (EC 1.1.1.21) and an invertase (EC 3.2.1.26), preferably a glycerol kinase and/or a transketolase.

4. The method for enhancing the erythritol productivity and/or yield of an erythritol-producing yeast strain according to claim 1, without producing erythrulose, characterized in that it further comprises inhibiting in said strain the expression or the activity of an endogenous erythritol dehydrogenase (EC 1.1.1.9) and optionally overexpressing in said strain at least one enzyme selected from the group consisting of a glycerol kinase (EC 2.7.1.30), a glycerol-3P dehydrogenase (EC 1.1.5.3), a triose isomerase (EC 5.3.1.1), a transketolase (EC 2.2.1.1), an erythrose 4 phosphate

phosphatase (EC 3.1.3.23), an erythrose reductase (EC 1.1.1.21) and an invertase (EC 3.2.1.26), preferably a glycerol kinase and/or a transketolase.

5. The method according to any one of claims 1 to 4, characterized in that the L-erythrulose kinase comprises or consists of the consensus amino acid sequence SEQ ID NO: 2.

6. The method according to any one of claims 1 to 4, characterized in that the L-erythrulose kinase is selected from the group consisting of SEQ ID NO: 1, 3, 4, 5 and 6.

7. The method according to any one of claims 1 to 6, characterized in that the yeast strain belongs to the genus selected from the group consisting of *Aurobasidium*, *Candida*, *Moniliella*, *Pseudozyma*, *Torula*, *Trichosporon*, *Trigonopsis* or *Yarrowia*, preferably *Yarrowia*.

8. The method according to claim 7, characterized in that the yeast strain is selected from the group consisting of *Y. lipolytica*, *Y. galli*, *Y. yakushimensis*, *Y. alimentaria* and *Y. phangnensis*, preferably *Y. lipolytica*.

9. The method according to any one of claims 1 to 8, characterized in that said inhibition is obtained by mutagenesis of the endogenous gene encoding said L-erythrulose kinase.

10. The method according to claim 9, characterized in that said inhibition is obtained by genetically transforming the yeast strain with a disruption cassette of said endogenous gene.

11. The method according to any one of claims 2 to 10, characterized in that said enzyme is endogenous or from a prokaryotic or eukaryotic organism.

12. The method according to any one of claims 2 to 11, characterized in that the glycerol kinase comprises or consists of the sequence SEQ ID NO: 8, the glycerol-3P dehydrogenase comprises or consists of the sequence SEQ ID NO: 9, the triose isomerase comprises or consists of the sequence SEQ ID NO: 10, the transketolase comprises or consists of the sequence SEQ ID NO: 11, and the erythrose reductase comprises or consists of the sequence SEQ ID NO: 12.

13. A method for enhancing the erythritol productivity and/or yield of an erythritol-producing yeast strain without production of erythrulose, characterized in that it comprises inhibiting in said yeast strain the expression or the activity of an

endogenous erythritol dehydrogenase (EC 1.1.1.9) having at least 50% identity or by order of increasing preference at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identity, with the polypeptide of sequence SEQ ID NO: 7 (YALI_EYD1) and optionally overexpressing in said strain at least one enzyme selected from the group consisting of a glycerol kinase (EC 2.7.1.30), a glycerol-3P dehydrogenase (EC 1.1.5.3), a triose isomerase (EC 5.3.1.1), a transketolase (EC 2.2.1.1), an erythrose 4 phosphate phosphatase (EC 3.1.3.23), an erythrose reductase (EC 1.1.1.21) and an invertase (EC 3.2.1.26), preferably a glycerol kinase and/or a transketolase.

14. A mutant erythritol and/or erythrulose-producing yeast strain, preferably a *Yarrowia* strain, wherein the expression or the activity of an endogenous L-erythrulose kinase is inhibited and optionally wherein at least one enzyme selected from the group consisting of an erythritol dehydrogenase (EC 1.1.1.9) and a glycerol kinase (EC 2.7.1.30), a glycerol-3P dehydrogenase (EC 1.1.5.3), a triose isomerase (EC 5.3.1.1), a transketolase (EC 2.2.1.1), an erythrose 4 phosphate phosphatase (EC 3.1.3.23), an erythrose reductase (EC 1.1.1.21), and an invertase (EC 3.2.1.26), preferably at least a glycerol kinase and/or a transketolase, is overexpressed.

15. A mutant erythritol-producing yeast strain without production of erythrulose, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of an endogenous L-erythrulose kinase and of an endogenous erythritol dehydrogenase is inhibited and optionally wherein at least one enzyme selected from the group consisting of a glycerol kinase (EC 2.7.1.30), a glycerol-3P dehydrogenase (EC 1.1.5.3), a triose isomerase (EC 5.3.1.1), a transketolase (EC 2.2.1.1), an erythrose 4 phosphate phosphatase (EC 3.1.3.23), an erythrose reductase (EC 1.1.1.21), and an invertase (EC 3.2.1.26), preferably at least a glycerol kinase and/or a transketolase, is overexpressed.

16. A mutant erythritol-producing yeast strain without production of erythrulose, preferably a *Yarrowia* strain, more preferably a *Y. lipolytica* strain, wherein the expression or the activity of an endogenous erythritol dehydrogenase is inhibited and optionally at least one enzyme selected from the group consisting of a glycerol kinase (EC 2.7.1.30), a glycerol-3P dehydrogenase (EC 1.1.5.3), a triose isomerase (EC 5.3.1.1), a transketolase (EC 2.2.1.1), an erythrose 4 phosphate phosphatase (EC

3.1.3.23), an erythrose reductase (EC 1.1.1.21), and an invertase (EC 3.2.1.26), preferably at least a glycerol kinase and/or a transketolase, is overexpressed.

17. Use of a mutant erythritol and/or erythrulose-producing yeast strain of any one of claims 14 to 16 for producing erythritol and/or erythrulose.

18. A method for producing erythritol and/or erythrulose, comprising a step of growing a mutant erythritol and/or erythrulose-producing yeast strain of any one of claims 14 to 16.

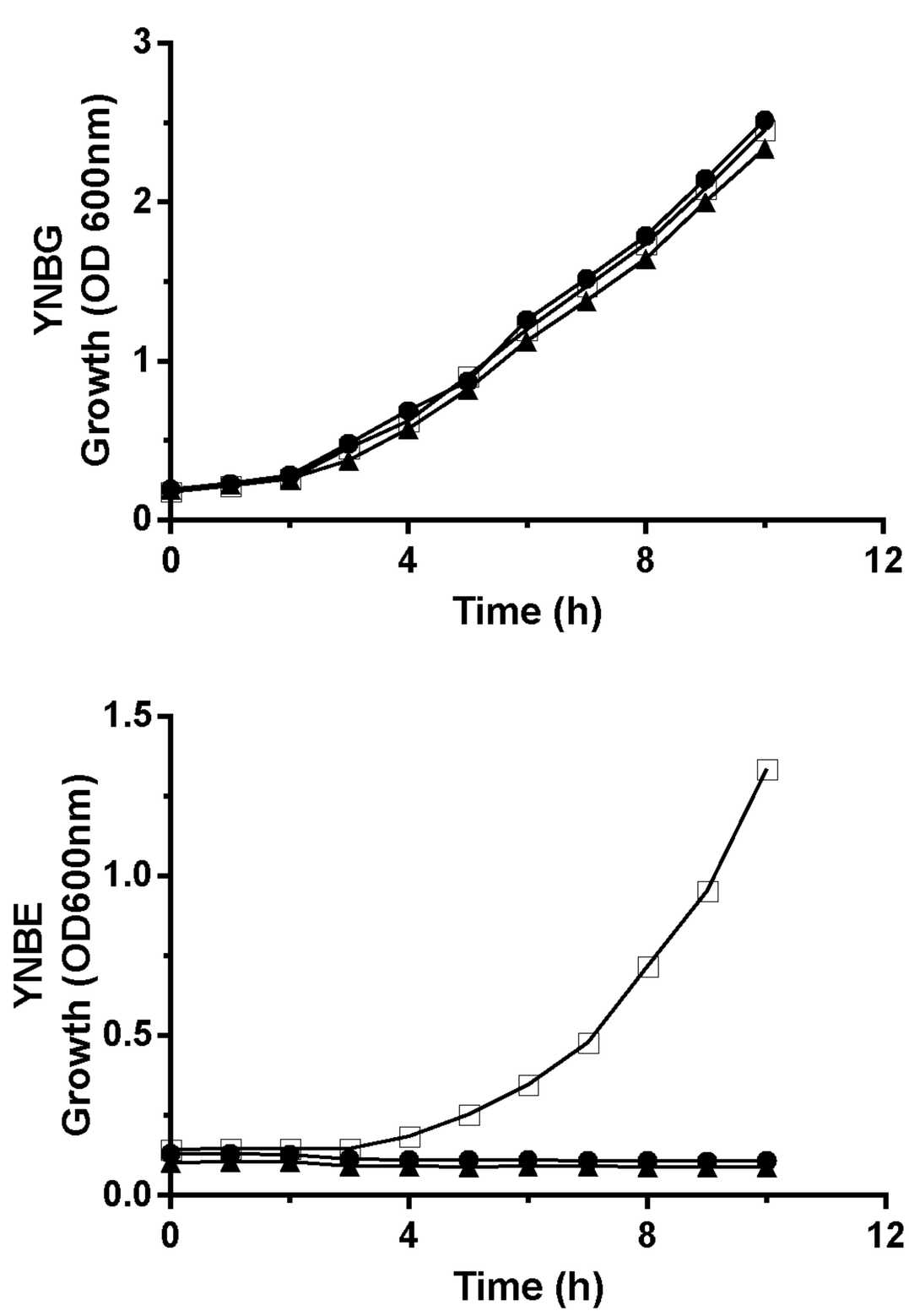

YNBG
Growth (OD 600nm)
0
1
2
3
0
4
8
12
Time (h)
YNBE
Growth (OD600nm)
0.0
0.5
1.0
1.5
0
4
8
12
Time (h)

Figure 1A

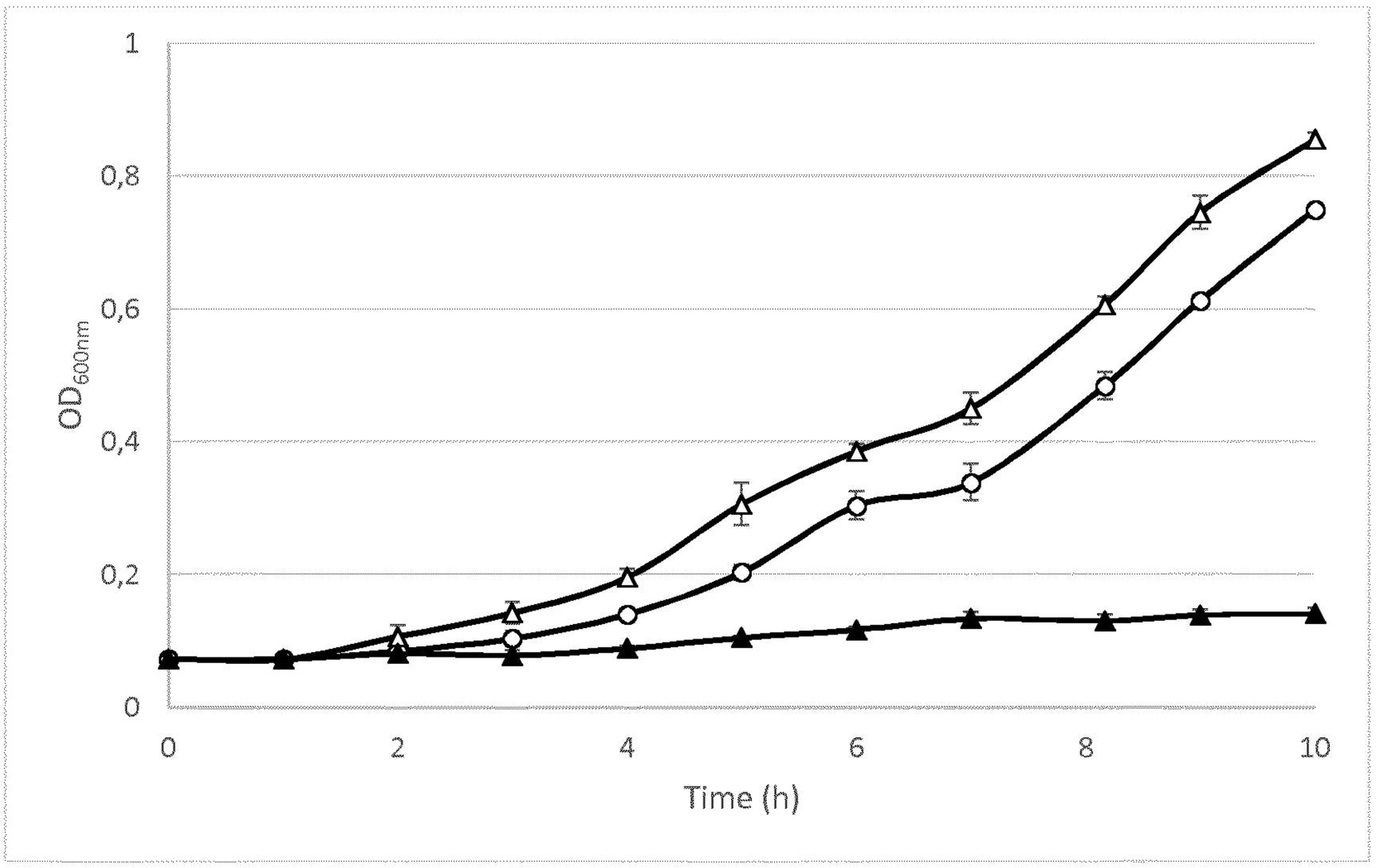


Figure 1B

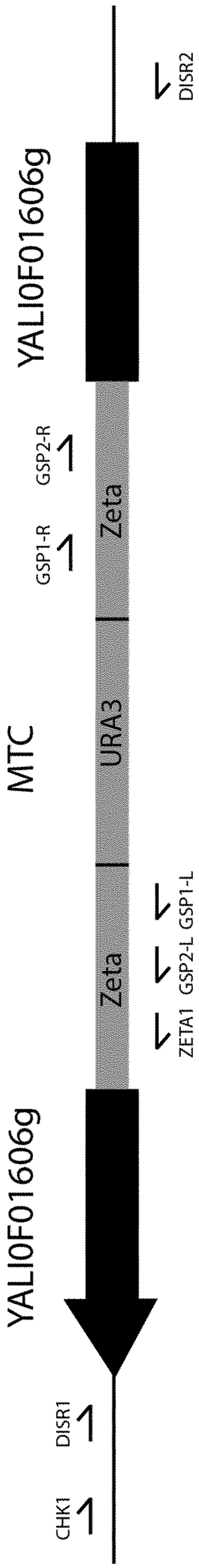

MTC
URA3
GSP1-R
GSP2-R
Zeta
YALI0F01606g
DISR2
Zeta
ZETA1
GSP2-L
GSP1-L
YALI0F01606g
DISR1
1
CHK1
1

Figure 2

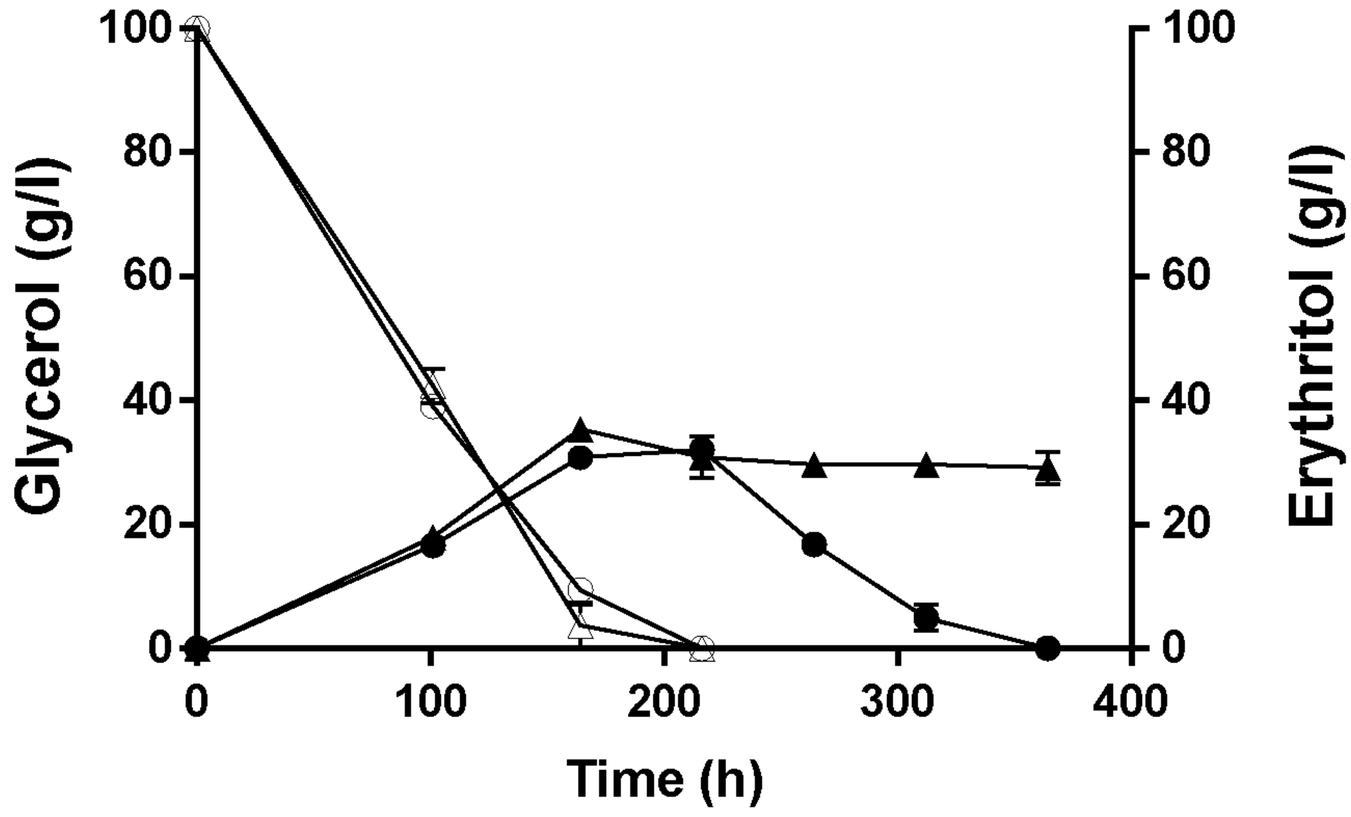


Figure 3A

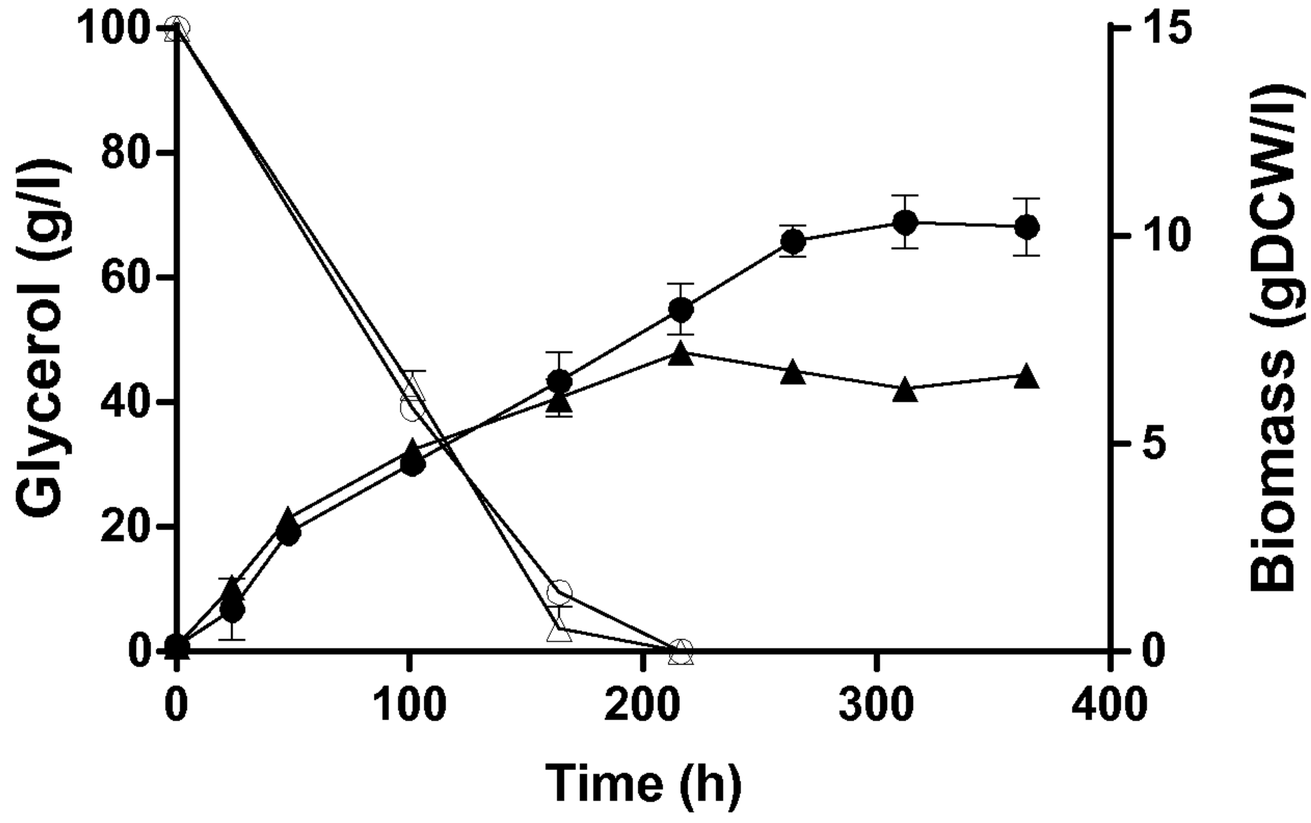


Figure 3B

```
YAPH_EYK1       MSTKHLFNETDDLVVKSLKGVQASRSASILSSRFKVLYNGLHTSERVAVLSGGGSGHEPA
YAAL_EYK1       MSTKHLFNETDELVLKSLEGVQASRSASILSNRFKVLYNGNHTKDRVAVLSGGGSGHEPA
YAYA_EYK1       MSTKHLFNETDELVLKSLEGVQASRQASILSHRFKVLYNGSHTSDRVAVLSGGGSGHEPA
YALI_EYK1       MSTKHLFNETDELVLKSLEGVQASRSASILSHRFKVLYNGTHSADRVAVLSGGGSGHEPA
YAGA_EYK1       MSTKHLFNETDELVLKSLEGVQASRSASILSHRFKVLYNGSHSADRVAVLSGGGSGHEPA
                ***********:**:***:******.***** ******** *: :***************

YAPH_EYK1       HAGFVGDNMLTGAICGPVFASPSAKQVEAGCKLVPSDKGHILVVTNYTGDMLHFGLAAEK
YAAL_EYK1       HAGFVGDNMLTGAICGPVFASPSAKQVEAGCKLVPSDKGHILVVTNYTGDMLHFGLAAEK
YAYA_EYK1       HAGFVGDNMLTGAICGPVFASPSAKQVEAGCKLVPSDKGHILVVTNYTGDMLHFGLAAEK
YALI_EYK1       HAGFVGDNMLTGAICGPVFASPSAKQVEAGCKLVPSDKGHILVVTNYTGDMLHFGLAAEK
YAGA_EYK1       HAGFVGDNMLTGAICGPVFASPSAKQVEAGCKLVPSDKGHILVVTNYTGDMLHFGLAAEK
                **************************************************************

YAPH_EYK1       LKAQGHNVGIIKSADDVAVDKKSGGLVGRRGLAGTVLLDKIVGAAAWDKLSFEECMEIGT
YAAL_EYK1       LKSQGHKVGIIKSADDVAVDRKSGGLVGRRGLAGTILLDKIVGAAAFAKLSFEECMEIGS
YAYA_EYK1       LKSQGHKVGIIKSADDVAVDRKSGGLVGRRGLAGTVLLDKIVGGAAWDKLSFDECMAIGT
YALI_EYK1       LKSQGHKVGIIKSADDVAVDRKSGGLVGRRGLAGTVLLDKIVGGAAWDKLSFDECMAIGT
YAGA_EYK1       LKSQGHKVGIIKSADDVAVDRKSGGLVGRRGLAGTVLLDKIVGGAAWDKLSFDECMAIGT
                **:***:*************.**************:*******.**: ****:*** **:

YAPH_EYK1       YVAENTATASIGLDFCHVPGRSVESHSSLGQNECQFGLGIHNEPGVKTLSPVPNPDTLIE
YAAL_EYK1       EVADNTATASIGLDYCHVPGRSVEKHSSLGQDECQFGLGIHNEPGVKTLSPIPSPDKLVE
YAYA_EYK1       EVSDNTATASIGLDYCHVPGRSVENHVSLVQDECQFGLGIHNEPGVKTLNPVPAPETMVR
YALI_EYK1       EVAENTATASIGLDYCHVPGRSVENHVSLDQNECQFGLGIHNEPGVKTINPVPAPESMVD
YAGA_EYK1       EVAENTATASIGLDYCHVPGRSVENHVSLAQEECQFGLGIHNEPGVKTMNPVPAPESMVE
                 *::**********:*********.* ** *:*****************:.*:* *:.::

YAPH_EYK1       TLLSYILSQDDPERSFVKFKENDEVILLANNLGGISTIEMRAAVLLAKEQLENTHNIKAV
YAAL_EYK1       TLLQYIVSQDDAQRSFVKFKEGDEVILLANNLGGISVIEMRAAVQLAVEQLEKTYKIASV
YAYA_EYK1       TLLDYLVSQDDHERSFVKFKQGDEVILLANNLGGISTIEMRAAVQLAREQLEKTHKIKPV
YALI_EYK1       TLLKYLVSQDDPERSFVKFKEGDEVILLANNLGGISTIEMRAAVQLAREQLEKTHKIKSV
YAGA_EYK1       TLLKYLVSQDDSERSFVKFKEGDEVILLANNLGGISTIEMRAAVQLAREQLEKTHKIKSV
                ***.*::**** :*******:.**************.******* ** ****:*::* .*

YAPH_EYK1       RVLCGTFMSSLNAPGFSLTLVNLSNGSHQNSVLKYLDAVSDAPAWVNVASPT-SVKPFVD
YAAL_EYK1       RVLCGTYMSSLNAPGFSLTLVNLSNGTHSKQVLEHLDATCDAPAWVNISPPV-SVKPFVS
YAYA_EYK1       RVLCGTFMSSLNAPGFSITLVNLSNGSHSNKVLQYLDAVSDAPAWVNVASPV-SVKPFVN
YALI_EYK1       RVLCGTFMSSLNAPGFSITLVNLSNGSHSKNVLKYLDAVSDAPAWVNVAPPT-SVKPFIN
YAGA_EYK1       RVLCGTFMSSLNAPGFSITLVNLSNGSHSKNVLKYLDAVSDAPAWVNTSPLTSSVKPFVS
                ******:**********:********:*.:.**::***..******* :. . *****:.

YAPH_EYK1       EECIFDEDY-SQVIAPTIPIDETELVSALTQASENIIKAEPQLTAWDTEMGDGDCGHTIE
YAAL_EYK1       EDTIFDESA-STLKEPVLDVDQKTLVAALKQASANIVKAEPQLTEWDTEMGDGDCGHTIE
YAYA_EYK1       EDKIFDDDI-SNLKAPNLDVSTQTVIAALTQASENIIKAEPQLTAWDTEMGDGDCGHTIE
YALI_EYK1       EDKIFDDET-SNIKAPTLDIPEQTVVAALTQASQNIIKAEPQLTAWDTEMGDGDCGHTIE
YAGA_EYK1       EDKIFDDETSSNIKAPVLDVPEQTIIAALTQASQNIIKAEPQLTAWDTEMGDGDCGHTIE
                *: ***:.  * :  * : :    :::**.*** **:******* ***************

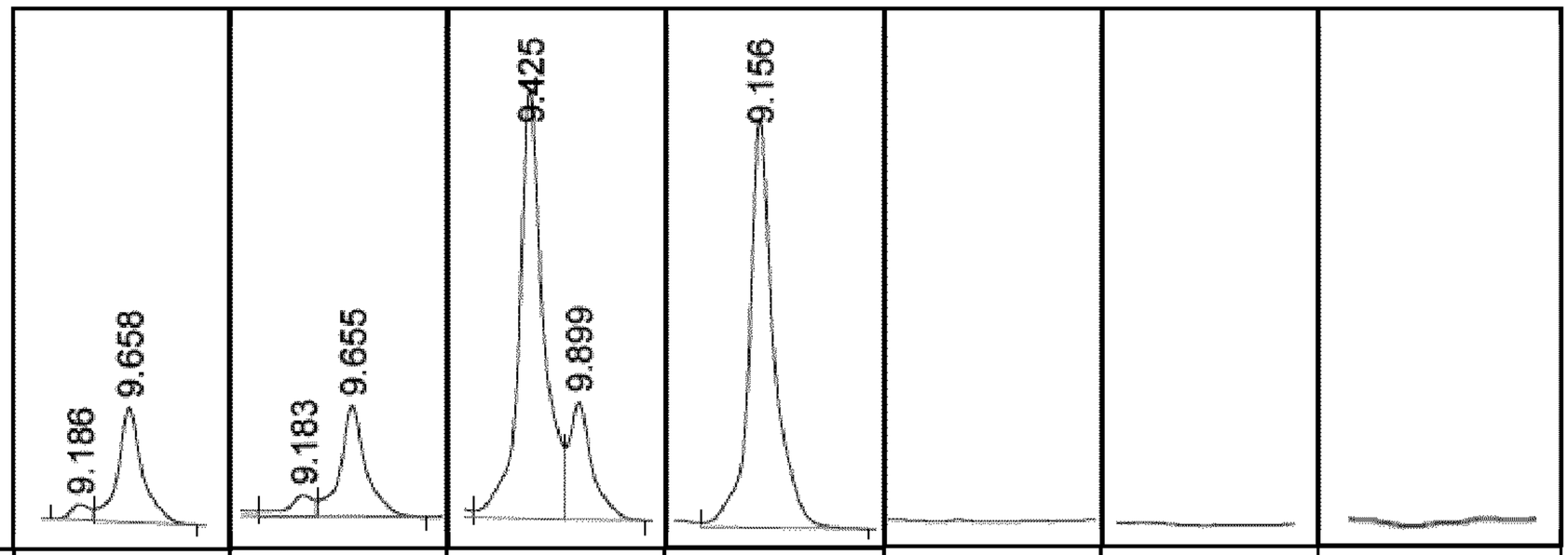
YAPH_EYK1       HGCRALLKYLENNKGNSEALKIIPIVREIVHITEEDMGGTLGAIFGIFFASFLNALLHDH
YAAL_EYK1       HGCKALVSYLDKNKNDPKALEIIPIVRAVVHITEEDMGGTLGAIFGIFFASFLNALLLDP
YAYA_EYK1       HGCRALLEYLHKNQNNTTALEIIPIVRDIVHITEEDMGGTLGAIFGIFFASFLNALLSDP
YALI_EYK1       HGCRALLEYLNKNKSDPKALEIIPIVRAVVHITEEDMGGTLGAIFGIFFASFLNALLLDP
YAGA_EYK1       HGCRALLEYLNKNKSDPKALEIIPIVRAVVHITEEDMGGTLGAIFGIFFASFLNALLLDP
                ***.**:.** :*:.:. **:****** :***************************** *
```

Figure 4a

```
YAPH_EYK1       CSHSPDVPVVEKLVNAANTGLESLMKHTPARPGDRTVMDVLIPYVQCLVATKNANEAAVK
YAAL_EYK1       LSHK-EVPVTDKLVNAANTGLESLMKHTPARPGDRTVMDVLIPYVQSLVATKDAKEAALK
YAYA_EYK1       LSHKTDVDVTDKLINAAHTGLESLMKHTPARPGDRTVMDVLIPYVTALVATKDVKEAALR
YALI_EYK1       LSHKTDVNVTDKLVNAANTGLESLMNHTPARPGDRTVMDVLIPYVQSLVSTKDIKEAALK
YAGA_EYK1       LSHKTDVSVTDKLVNAANTGLESLMNHTPARPGDRTVMDVLIPYVQSLVATKDIKEAALK
                 **. :* *.:**:***:*******:********************* .**:**: :***:.

YAPH_EYK1       AKEAAEGTKKIKPRLGRAVYVGEKDGELPPDPGAWAVYELVDGLSNRK
YAAL_EYK1       AKQAAEGTKKIKPRLGRAVYVGEKDGELPPDPGAWAVFELVDGFANHQ
YAYA_EYK1       AKQAAEGTKKIKPRLGRAVYVGEKDGELPPDPGAWAVYELVDGFANY-
YALI_EYK1       AKQAAEGTKKIKPRLGRAVYVGEKDGELPPDPGAWAVYELVDGFANHK
YAGA_EYK1       AKQAAEGTKKIKPRLGRAVYVGEKDGELPPDPGAWAVYELVDGFANHK
                **:*********************************:******::*
```

Figure 4b



Strain	FCY001	FCY001	FCY001	-	-	FCY001	JMY2900
Medium	ERY	ERY	ERY	-	-	GLU	ERY
Erythrulose	-	-	+	+	-	-	-
Erythritol	-	+	-	-	+	-	-

Figure 5

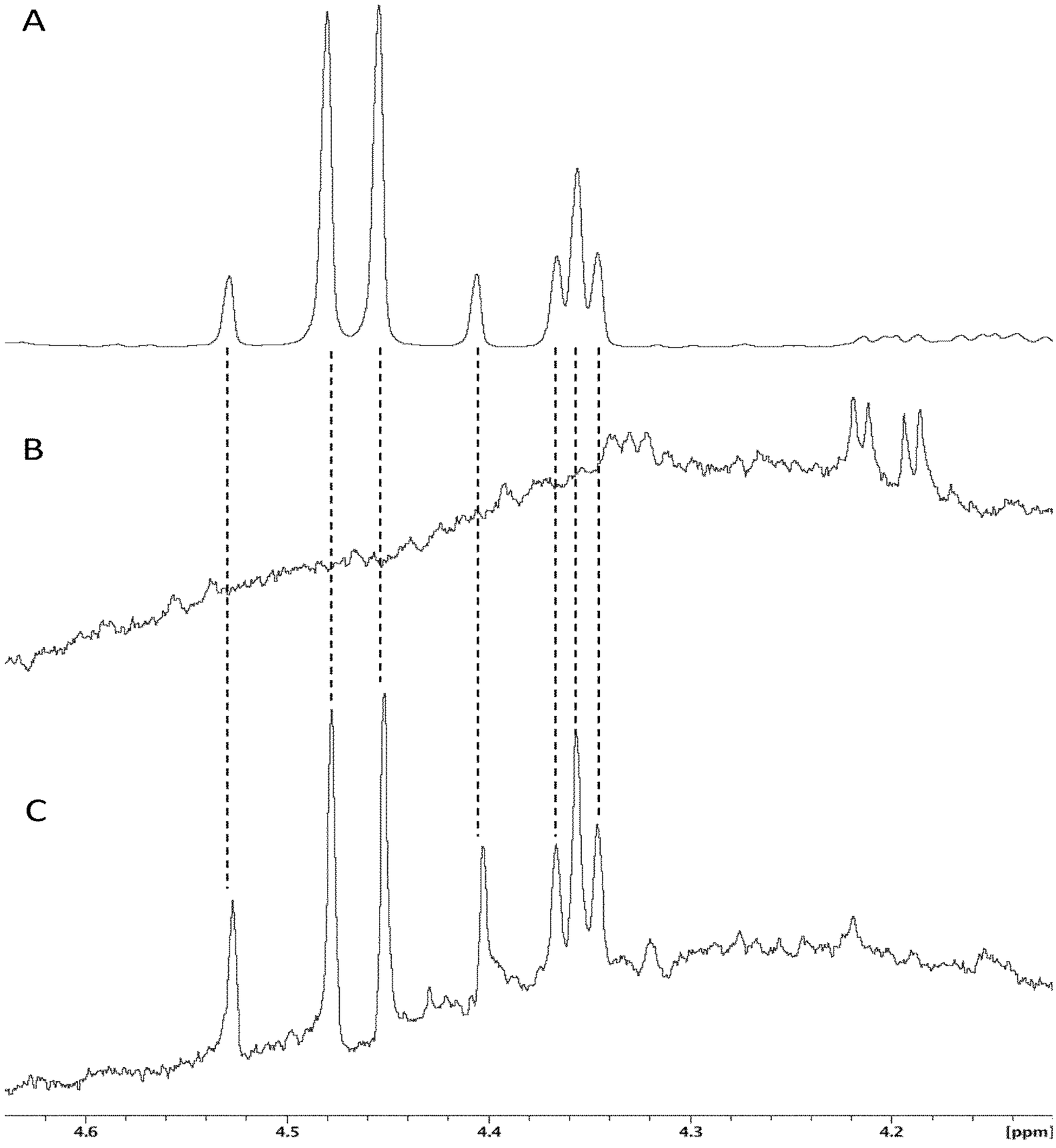


Figure 6

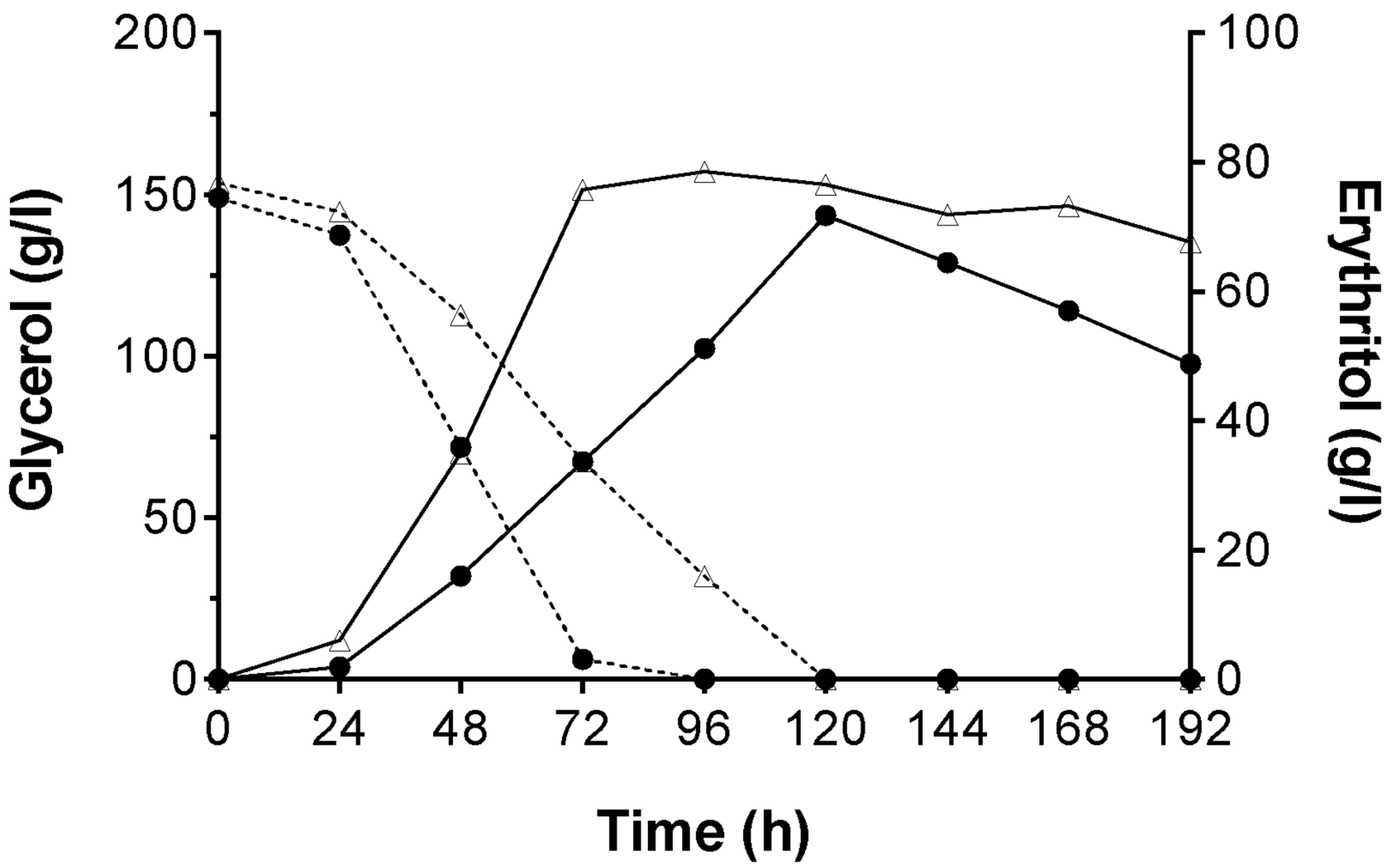

Glycerol (g/l)
Erythritol (g/l)
Time (h)
0
50
100
150
200
0
20
40
60
80
100
0
24
48
72
96
120
144
168
192

Figure 7

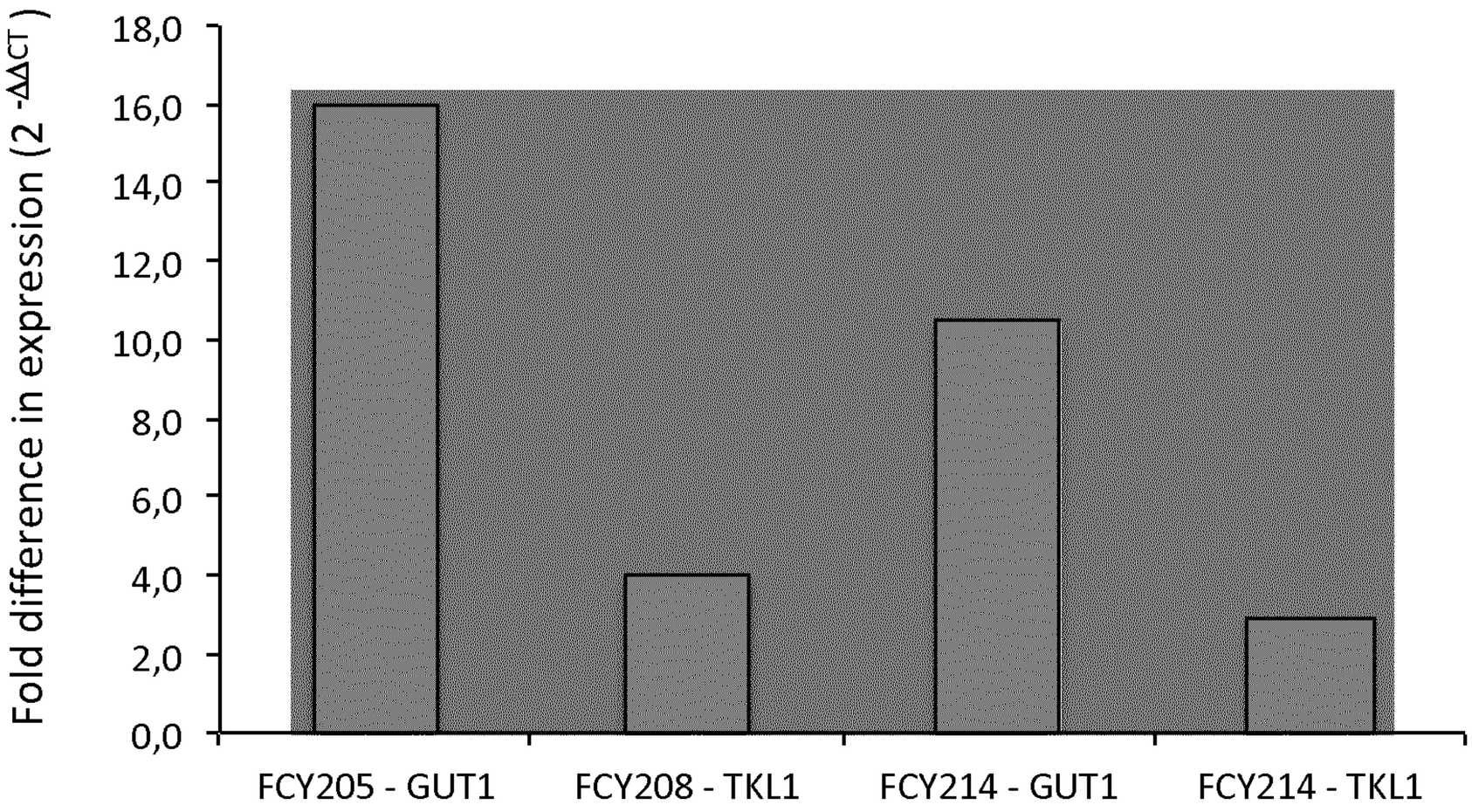

Fold difference in expression (2 -ΔΔCT)
18,0
16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
FCY205 - GUT1
FCY208 - TKL1
FCY214 - GUT1
FCY214 - TKL1

Figure 8