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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 December 2011 (08.12.2011)

PCT

(10) International Publication Number
WO 2011/151326 A2

(51) International Patent Classification:
A23L 1/23 (2006.01)

(21) International Application Number:
PCT/EP2011/058949

(22) International Filing Date:
31 May 2011 (31.05.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

10164511.7	31 May 2010 (31.05.2010)	EP
61/465,803	23 March 2011 (23.03.2011)	US

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, 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, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

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

WO 2011/151326 A2

(54) Title: THE USE OF TRANSPORTERS TO MODULATE FLAVOR PRODUCTION BY YEAST

(57) Abstract: The present invention relates to the use of transporters to modulate production of flavor and metabolically related compounds by yeast. More specifically, it related to the use of transporters of the ATP binding cassette (ABC) transporter family, the oligopeptide transporter (OPT) superfamily or the Major Facilitator Superfamily (MFS) to increase or decrease secondary metabolites linked to the isoleucine / valine pathway, such as, but not limited to α-acetolactate, diacetyl, pentanedione, iso-butyl alcohol, iso-amyl alcohol and isoamyl acetate.

THE USE OF TRANSPORTERS TO MODULATE FLAVOR PRODUCTION BY YEAST

The present invention relates to the use of transporters to modulate flavor production by yeast. More specifically, it related to the use of transporters of the ATP binding cassette (ABC) transporter family, the oligopeptide transporter (OPT) superfamily or the Major Facilitator Superfamily (MFS) to increase or decrease secondary metabolites linked to the isoleucine / valine pathway, such as, but not limited to α-acetolactate, diacetyl, pentanedione, iso-butyl alcohol, iso-amyl alcohol and isoamylacetate.

Saccharomyces spp are widely used for the production of food and beverages, such as bread, beer and wine. The yeast produces a range of flavor products which are essential for the taste of this fermented food and beverages. However, the flavor balance is delicate and difficult to control; essential flavor compounds can become off flavors in high concentrations. Moreover, production and removal of unwanted flavors, such as diacetyl in beer, may determine the fermentation time. A more efficient control of flavor production would allow a better quality control, and could help to increase the productivity in some fermentations.

In a classical fermentation process, flavor production is an interplay between the yeast strain used and the fermentation conditions. Adaptation of the fermentation conditions, however, may influence several flavors, and allows only flavor control to a limited extend. A typical example is the production of the off flavor diacetyl in beer production. Diacetyl has a buttery taste that is strongly unwanted in lager beers. Diacetyl is produced as a by-product of the isoleucine/valine pathway. α-acetolactate, an intermediate of the isoleucine/valine pathway, is secreted in the medium by the yeast. By spontaneous chemical decarboxylation, diacetyl is formed. The uptake of diacetyl by the yeast and the enzymatic reduction of diacetyl to the flavor inactive compound acetoin is determining the maturation period of the beer.

The chemical decarboxylation is the rate limiting step in the process. One can speed up diacetyl formation and consequent diacetyl uptake and reduction by increasing the temperature during maturation; however, a higher maturation temperature may lead to other unwanted compounds such as sulphury flavors and fatty acids due to yeast lysis. Alternatively, the addition of a bacterial α-acetolactatedecarboxylase to the fermenting wort has been proposed. Although technically feasible, addition of an enzyme is increasing the fermentation cost, and increases the risk of contamination.

Surprisingly we found that modulating the expression level of one or more transporters is influencing the flux through the isoleucine/valine pathway, and particularly the secretion of isoamyl acetate, pentanedione, isobutanol and α-acetolactate. This is particularly surprising, as no transporters for those compounds have been described in yeast, and several of the

active transporters have been identified as being involved in transport of unrelated compounds.

A first aspect of the invention is the use of a transporter which is not an amino acid permease (pfam00324) to modulate the production of byproducts of the isoleucine/valine pathway, preferably byproducts selected from the group consisting of diketones, higher alcohols and esters derived from higher alcohols. Preferably, said byproducts are flavour active, i.e. determining the flavor of the end product at low concentrations. Preferably, said byproducts are selected from the group consisting of diacetyl, pentanedione, isoamyl acetate and isobutanol. Preferably said transporter is selected from the group consisting of the oligopeptide transporter (OPT) superfamily, the Major Facilitator superfamily (MFS) and the ATP binding cassette (ABC) transporters. Even more preferably said transporter is a yeast member of said families. A yeast, as used here, is a unicellular, non-filamentous fungus. Yeasts are known to the person skilled in the art, and include, but are not limited to the genera *Saccharomyces, Schizosaccharomyces, Zygosaccharomyces, Kluyveromyces, Brettanomyces, Hansenula, Pichia,* and *Yarrowia.* Most preferably, said yeast is a *Saccharomyces* sp. The use of a transporter according to the invention can be combined with the use of one or more other transporters according to the invention.

*Saccharomyces m*embers of the OPT superfamily comprise the transporters encoded by the genes *OPT1* (SEQ ID NO: 1) and *OPT2 (HTG1)* (SEQ ID NO: 2) and homologs and variant thereof; orthologs of those genes can be identified in other yeast genera. ABC transporters as used here include proteins catalogued in the MIPS FUNCAT as 20.03.25 – ABC transporters (Ruepp et al., 2004). *Saccharomyces* ABC transporters are known to the person skilled in the art and include, but are not limited to the proteins encoded by *ADP1 (SEQ ID NO: 3), ARB1 (SEQ ID NO: 4), ATM1 (SEQ ID NO: 5), ATR1 (SEQ ID NO: 6), AUS1 (SEQ ID NO: 7), BPT1 (SEQ ID NO: 8), ENB1 (SEQ ID NO: 9), MDL1 (SEQ ID NO: 10), MDL2 (SEQ ID NO: 11), NFT1 (SEQ ID NO: 12), PDR5 (SEQ ID NO: 13), PDR10 (SEQ ID NO: 14), PDR11 (SEQ ID NO: 15), PDR12 (SEQ ID NO: 16), PDR15 (SEQ ID NO: 17), PDR18 (SEQ ID NO: 18), PXA1 (SEQ ID NO: 19), PXA2 (SEQ ID NO: 20), QDR2 (SEQ ID NO: 21), RLI1 (SEQ ID NO: 22), SNQ2 (SEQ ID NO: 23), STE6 (SEQ ID NO: 24), VMR1 (SEQ ID NO: 25), YBT1 (SEQ ID NO: 26), YCF1 (SEQ ID NO: 27), YOR1 (SEQ ID NO: 28), YKR104W (SEQ ID NO: 29) and YOL075c (SEQ ID NO: 30)*; orthologs of those genes can be identified in other yeast genera. Members of the MFS are known to the person skilled in the art, and belong to the MFS clan (CL0015) in the pfam database. As used here, they include, but are not limited to the *Saccharomyces* transporters encoded by *AQR1 (SEQ ID NO: 31), AZR1 (SEQ ID NO: 32), DTR1 (SEQ ID NO: 33), FLR1 (SEQ ID NO: 34), HOL1 (SEQ ID NO: 35), NFT1 (SEQ ID NO: 12), QDR1 (SEQ ID NO: 36), QDR2 (SEQ ID NO: 21), QDR3 (SEQ ID NO: 37), SEO1 (SEQ*

ID NO: 38), SGE1 (SEQ ID NO: 39), THI7 (SEQ ID NO: 40) and *VBA5 (SEQ ID NO: 41)* and homologs, orthologs or variants thereof. Homologs, orthologs and variants of these transporters are known to the person skilled in the art, and include, but are not limited to protein sequences of other yeast genera with a high sequence identity (preferably at least 70% identities, more preferably at least 80% identities, most preferably at least 90% identities as measured in a BLASTp (Altschul et al., 1997; Altschul et al., 2005) and a similar function. Preferably a homolog, ortholog or variant, as defined here, is a sequence that can complement a knock out mutation of the homologous, orthologous or variant sequence in *Saccharomyces* sp.

Preferably, said transporter is selected from the group consisting of Aqr1p, Pdr12p, Opt1p, Adp1p and Qdr2p, or a homolog or variant thereof. Most preferably, said transporter is Opt1p, optionally in combination with another transporter. Aqr1p is thought to be involved in the secretion of amino acids (Velasco et al., 2004). Pdr12p is described to be involved in the export of aromatic and branched chain organic acids, but not in the fusel alcohols derived from the isoleucine – valine pathway (Hazelwood et al., 2006). Opt1p is normally involved in oligopeptide, glutathione and phytochelatin transport (Wiles et al., 2006, Bourbouloux et al.,2000). Qdr2p is implicated in potassium uptake (Vargas et al., 2007). Adp1p is a member of the MFS, but its specific function is unknown. None of those transporters have been linked to the secretion of diacetyl, pentadione, isoamyl acetate or isobutanol.

Said use is an upregulation or a downregulation of the activity of the transporter. Upregulation of the activity of the transporter, as used here, is reflected by an increase in the total activity of the transporter, compared with a reference strain grown in the same conditions, and can be, but is not necessarily realized by an increase of messenger RNA or protein, but might be due to an increase in specific activity of the protein. In the same way, downregulation, as used here, is reflected by a decrease in total activity of the receptor compared with a reference strain grown in the same conditions, and is not necessarily realized by a decrease of messenger RNA or protein, but might be due to a decrease in specific activity of the protein. As a non limiting example, upregulation by overexpression can be realized by placing the coding sequence of the transporter under control of a strong promoter, preferably a strong constitutive promoter. The choice of the strong promoter will depend on the organism for which it is used. Strong promoters for most industrial organisms, such as for *Saccharomyces* spp. are known to the person skilled in the art. Alternatively, overexpression is realized by increasing the copy number of the gene encoding the transporter. This can be realized by transforming several copies of a gene encoding an endogenous transporter into a host, or it can be realized by the transformation of one of more copies of a gene encoding a heterologous transporter into the host. Upregulation can also be obtained by random

mutagenesis and selection. Methods to obtain downregulation are known to the person skilled in the art and include, but are not limited to knock out constructs, in which the coding sequence, the promoter of the gene of both are inactivated or removed. Inactivation of the coding sequence can be realized by the insertion of one or more nonsense codons, resulting in an inactive protein. The promoter region can be deleted as a whole, or partially, or the promoter can be replaced by a weaker promoter or by a promoter that is conditionally expressed. For the use of the invention, every mutation that is upregulating or downregulating the activity of a transporter according to the invention can be used. Methods for creating mutations are well known to the person skilled in the art and include but are not limited to site directed mutagenesis, random mutagenesis and selection or selection of spontaneous mutations. Chemical mutagenesis of micro-organisms, including yeast commonly involves treatment of yeast cells with DNA mutagens such as, but not limited to ethylmethanesulfonate (EMS), nitrous acid, diethyls sulphate or N-methyl-N'-nitro-N-nitroso-guanidine (MNNG). Irradiation with ultraviolet light (UV) or X-ray can also be used to produce random mutagenesis in yeast cells. Selection of mutations can be based for instance on screening of the activity of the transporter, or on specific amplification by PCR of the sequence to be mutated, optionally followed by sequencing of the fragment. Alternatively, downregulation of a transporter can be screened by the acquirement of resistance to a toxic analogue taken up by the relevant transporter. Alternatively, the mutation may be introduced in a certain strain by crossing this strain with another strain carrying the mutation, possibly followed by segregation of the characteristic and selection for the mutation. Another possibility of downregulating the expression is by the use of antisense RNA or by RNAi. Still another possibility is the use of inhibitors of the transporter in the medium, which can block the activity of the transporter. Upregulation and downregulation can also be realized by adapting the medium, i.e. by adding a compound that is normally not present in the medium, or not present at the concentration used for up- or downregulation. As a non-limiting example, *OPT1 (protein: SEQ ID NO: 1)* can be downregulated by adding sulfur to the medium (Wiles et al., 2006)

Preferably, said upregulation or downregulation is realized in a yeast, preferably in a *Saccharomyces* sp. Preferably, the use according to the invention is a downregulation. Even more preferably said yeast is a polyploid or an aneuploid yeast, preferably a wine yeast, distiller's yeast or brewer's yeast. Most preferably, said yeast is a brewer's yeast. Polyploid and aneuploid yeasts are known to the person skilled in the art and refer to yeast with more than two times the number of haploid chromosomes (n). In a polyploidy strain, all chromosomes are represented in each set (3n, 4n, 5n – Xn, with X>2...); in an aneuploid strain, some chromosomes are overrepresented (Xn + Y, with $X \geq 2$, and Y >0). Brewer's yeast, as used here, is a yeast suitable for beer production, preferably a yeast used in

industrial beer production. It can be a lager or bottom fermenting yeast, or an ale or top fermenting yeast. In a similar way, a wine yeast is a yeast suitable for wine production, preferably used in an industrial wine production, and a distillers yeast is a yeast suitable for the fermentation during the production of a distilled beverage, preferably a yeast used in the industrial production of a distilled beverage.

Another aspect of the invention is a method for limiting diacetyl production during a *Saccharomyces* spp. fermentation comprising at least (1) downregulation of one or more transporters encoded by the genes selected from the group consisting of *AQR1 (protein: SEQ ID NO: 31), PDR12 (protein: SEQ ID NO: 16) and OPT1 (protein: SEQ ID NO: 1)*, and (2) using the strain with the downregulated transporter(s) in a fermentation process. In one preferred embodiment, said fermentation process is a wine fermentation. In another preferred embodiment, said fermentation process is a beer fermentation. In one preferred embodiment, said *Saccharomyces* sp. is a bottom fermenting yeast. In another preferred embodiment, said *Saccharomyces* sp. is a top fermenting yeast. "Limiting diacetyl production" as used here, means that the amount of diacetyl produced by the yeast during and/or at the end of the fermentation is lower for the strain comprising the downregulated transporter, as compared with the parental strain when used under comparable fermentation conditions. The amount of diacetyl produced is evaluated as described in the materials and methods, and the result of the equilibrium of alpha-acetolactate secretion and diacetyl uptake and reduction.

Another aspect of the invention is a method for increasing isoamyl acetate production during a *Saccharomyces* sp. fermentation, comprising at least (1) downregulation of one or more transporters encoded by the genes selected from the group consisting of *PDR12 (protein: SEQ ID NO: 16), OPT1 (protein: SEQ ID NO: 1) and QDR2 (protein: SEQ ID NO: 21)*, and (2) using the strain with the downregulated transporter(s) in a fermentation process. In one preferred embodiment, said fermentation process is an alcoholic fermentation, such as beer, wine, or alcoholic spirit fermentation. In the latter case, the flavor production during fermentation will contribute to the flavor of the distilled beverage. In another preferred embodiment, said fermentation process is a solid state fermentation. Indeed, yeasts are known to contribute to the flavor of several products produced by solid state fermentation. As a non-limiting example, yeasts are used in the solid state fermentation of bread dough, but also of wheat bran, corn grits, eastern rice wines and cacao beans.

Still another aspect of the invention is a method for increasing isobutanol production during a *Saccharomyces* sp. fermentation, comprising at least (1) downregulation of Opt1p, possibly in combination with the downregulation of another transporter, and (2) using the strain with the downregulated transporter(s) in a fermentation process. Due to its higher energy content, the

production of isobutanol may be a valuable alternative for bio-ethanol fermentation; however, the yield of isobutanol production by *Saccharomyces* is too low. The method of the invention is solving or helping to solve this problem. Production of isobutanol is not limited to use as a biofuel. Isobutanol can also serve as an important platform chemical for the production of other compounds useful in agricultural, biomedical, pharmaceutical or other applications.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Diacetyl concentration in the fermentation broth at the end of the fermentation for the wild type strain and the different transporter mutants.

Figure 2: Pentandione concentration in the fermentation broth at the end of the fermentation for the wild type strain and the different transporter mutants.

Figure 3: Isoamyl acetate concentration in the fermentation broth at the end of the fermentation for the wild type strain and the different transporter mutants.

Figure 4: Isobutanol concentration in the fermentation broth at the end of the fermentation for the wild type strain and the different transporter mutants.

Figure 5: Diacetyl concentration in the fermentation broth at the end of the fermentation for the wild type strain and the different single, double and triple transporter mutants.

Figure 6: Pentandione concentration in the fermentation broth at the end of the fermentation for the wild type strain and the different single, double and triple transporter mutants.

EXAMPLES

Materials and methods to the examples

Strains

Strains used:

Strain	Genotype	Source
BY4741 *Δaqr1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 aqr1Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 *Δpdr12*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pdr12Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 *Δopt1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 opt1Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 *Δadp1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 adp1Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 *Δaus1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 aus1Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 *Δtpo1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 tpo1Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 *Δtpo4*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 tpo4Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 *Δpdr10*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pdr10Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 *Δsnq2*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snq2Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 *Δqdr2*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 qdr2Δ0::KAN*^{r}	ResGen/ Invitrogen Belgium
BY4741 (wt)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen/ Invitrogen Belgium

The strains, carrying a single mutation were used to carry the double and triple mutations were constructed using plasmids from the DEL-MARKER-SET from Euroscarf. Mutations are introduced using the loxP-KanMX-loxP cassette from pUG6 (Güldener et al., 1996) or with the help of pUG66 (Güldener et al, 2002); the marker can be removed using Cre-recombinase, and pSH47 (Güldener et al, 1996).

Fermentations

Yeast precultures were shaken overnight at 30°C in test tubes with 5 ml of 8% YPD medium. After 16 h of growth, 1 ml of the overnight culture was used to inoculate 50 ml of 8% YPD medium in 250 ml Erlenmeyer flasks, and this second preculture was shaken at 30°C until stationary growth phase was reached. Cells were washed with sterile, distilled water, and used

to inoculate 350 ml of fresh, prewarmed (20°C) 12% YPD medium. Yeast strains were inoculated at 5 million cells per ml. Static fermentation was carried out at 20°C in flasks with water locks placed on top, in order to create semi-anaerobic conditions mimicking full-scale fermentations. Samples for chromatographic analysis were taken when fermentation was completed and immediately cooled on ice in an airtight container.

Analysis of volatile compounds

Headspace gas chromatography (HS-GC) coupled with flame ionization detection (GC-FID) was used for the measurement of acetate esters, ethyl esters and higher alcohols in the fermentation products. Headspace gas chromatography coupled with electron capture detection (GC-ECD) was used for the measurement of vicinal diketones in the fermentation products. Fermentation samples were cooled on ice, centrifuged and filtered, after which 5 ml was collected in vials, which were immediately closed. Before analysis, the samples were heated at 60°C for 1 h to cause complete conversion of α-acetolactate into diacetyl. Samples were then analyzed with a calibrated Autosystem XL gas chromatograph with a headspace sampler (HS40; Perkin-Elmer, Wellesley, MA, USA) and equipped with a CP-Wax 52 CB column (length, 50 m; internal diameter, 0.32 mm; layer thickness, 1.2 µm; Chrompack, Varian, Palo Alto, CA, USA). Samples were heated for 25 min at 70°C in the headspace autosampler before injection (needle temperature: 105°C). Helium was used as the carrier gas. The oven temperature was held at 50°C for 5 min, then increased to 200°C at a rate of 5°C per min and finally held at 200°C for 3 min. The FID and ECD temperatures were kept constant at 250°C and 200°C, respectively. Analyses were carried out in duplicate, and the results were analyzed with Perkin-Elmer Turbochrom Navigator software. To determine the end values of the aroma compounds, the results were recalculated to 5% (v/v) ethanol to normalize.

Example 1: Analysis of diacetyl production during fermentation

Transporters were inactivated by inserting the kanamycin resistance gene. Fermentations with the transporter mutants and with wild type as reference were set up as described in the materials and methods. At the end of the fermentation, the diacetyl and pentandione concentration was measured. The results are summarized in figure 1 and 2. A decrease in diacetyl production was measured in several mutants; the decrease was most pronounced in the deletions of *AQR1, PDR12, OPT1, ADP1 and QDR2.* Those results were confirmed by the analysis of the pentandione concentration, showing a similar trend as the diacetyl concentration.

Example 2: Production of esters and higher alcohols in the transporter mutants

Beside diacetyl and pentanedione, other volatile compounds, including acetaldehyde, ethyl acetate,isoamyl alcohol, isoamyl acetate, isobutanol, ethyl hexanoate and ethyl octanoate were analyzed at the end of the fermentation. In all cases, an effect (decrease or increase) of the mutation of the transporter on the volatile production can be demonstrated. The results for isoamyl acetate and isobutanol are summarized in figure 3 and 4.

Example 3: effect of double and triple mutations

Several transporter mutations were combined to study the effect of multiple mutations on the flavor profile. Fermentations and analysis of the volatiles was carried out as described above.

A clear effect could be noticed on the concentration of acetaldehyde, ethyl acetate, propanol, isobutanol, iso amyl acetate, iso amyl alcohol, ethyl hexanoate, ethyl octanoate, diacetyl and pentanedion. The results for diacetyl and pentanedion are shown in figure 5 and figure 6, respectively. A decrease of about 35% in diacetyl production was seen for the *aqr1 / pdr12* double mutant. Similar results in reduction of diacetyl production are obtained for the same transporter mutants using another parental *Saccharomyces* strain.

Production of isobutanol was highest in the *aqr1 / opt1 / pdr12* triple mutant (increase in isobutanol concentration at the end of the fermentation of about 50%)

REFERENCES

- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.L. (1997), Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25, 3389-3402.
- Altschul, S.F., Wootton, J.C., Gertz, E.M., Agarwala, R., Morgulis, A., Schäffer, A.A. and Yu, Y.K. (2005). Protein database searches using compositionally adjusted substitution matrices, FEBS J. 272, 5101-5109.
- Bourbouloux A., Sahi, P., Chakladar, A., Delrot, S and Bachhawat, A.K. (2000). Hgt1p, a high affinity gluthathione transporter from the yeast *Saccharomyces cerevisiae.* J. Biol. Chem. 275, 13259-13265.
- Güldener, U., Heck, S., Fiedler, T., Beinhauer, J. and Hegemann, J.H. (1996). A new efficient disruption cassette for repeated use in budding yeast. Nucleic Acids Research 24, 2519-2524.
- Güldener, Y., Heinisch, J., Köhler, G.J., Voss, D. And Hegemann, J.H. (2002). A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeasts. Nucleid acids Research, 30, e23.
- Hazelwood, L.A., Tai, S.L., Boer, V.M., de Winde, J.H., Pronk, J.T. and Daran, J.M. (2006). A new physiological role for Pdr12p in *Saccharomyces cerevisiae*: export of aromatic branched chain organic acids produced in amino acid catabolism. FEMS Yeast res. 6, 937-945.
- Ruepp, A., Zollner, A., Maier, D., Albermann, K., Hani, J., Mokrejs, M., Tetko, I., Güldener, U., Mannhaupt, G., Münsterkötter, M. and Mewes, H.W. (2004). The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucl. Acids Res. 32, 5539-5545.
- Vargas, R.C., Garcia-Salcedo, R, Tenreio, S., Teiseira, M.C., Fernandes, A.R., Ramos, J and Sa-Correia, I. (2007) *Saccharomyces cerevisiae* multidrug resistance transporter Qdr2 is implicated in potassium uptake, providing a physiological advantage to quinidine-stressed cells. Eukaryot. Cell 6, 134-142.
- Velasco, I., Tenreiro, S., Calderon, I.L. and André, B. (2004). *Saccharomyces cerevisiae* Aqrl is an internal-membrane transporter involved in excretion of amino acids. Eukaryotic Cell, 3, 1492-1503.
- Wiles, A.M., Cai, H., Naider, F. and Becker, J.M. (2006). Nutrient regulation of oligopepetide transport in *Saccahromyces cerevisiae.* Microbiology 152, 3133-3145.

CLAIMS

1. The use of a transporter, selected from the group consisting of the oligopeptide transporter (OPT) superfamily, the Major Facilitator superfamily (MFS) and the ATP binding cassette (ABC) transporters to modulate the production of by-products of the isoleucine / valine pathway.
2. The use of a transporter according to claim 1, whereby said by-product is selected from the group consisting of diketones, higher alcohols, or esters derived from higher alcohols.
3. The use of a transporter according to claim 1 or 2, whereby said by-product is selected from the group consisting of diacetyl, pentandione, isoamyl acetate and isobutanol.
4. The use of a transporter according to any of the preceding claims, whereby said transporter is a yeast transporter.
5. The use of a transporter according to claim 4, whereby said transporter is a *Saccharomyces* sp. transporter.
6. The use of a transporter according to claim 5, whereby said transporter is encoded by a gene selected from the group consisting of AQR1 (SEQ ID NO: 31), PDR12 (SEQ ID NO: 16), OPT1 (SEQ ID NO: 1), ADP1 (SEQ ID NO: 3) and QDR2 (SEQ ID NO: 21).
7. The use of a transporter according to any of the previous claims, whereby the activity of said transporter is upregulated or downregulated in yeast.
8. The use of a transporter according to any of the previous claims, whereby said yeast is *Saccharomyces* sp.
9. A method for limiting diacetyl production during a *Saccharomyces* sp. fermentation, comprising at least (1) downregulation of one or more transporters encoded by a gene selected from the group consisting of AQR1 (SEQ ID NO: 31), PDR12 (SEQ ID NO: 16) and OPT1 (SEQ ID NO: 1), and (2) using the strain with the downregulated transporter(s) in a fermentation process.
10. A method for increasing isoamyl acetate production during a *Saccharomyces* sp. fermentation, comprising at least (1) downregulation of one or more transporters encoded by a gene selected from the group consisting of PDR12 (SEQ ID NO: 16), OPT1 (SEQ ID NO: 1) and QDR2 (SEQ ID NO: 21), and (2) using the strain with the downregulated transporter(s) in a fermentation process.

Figure 1

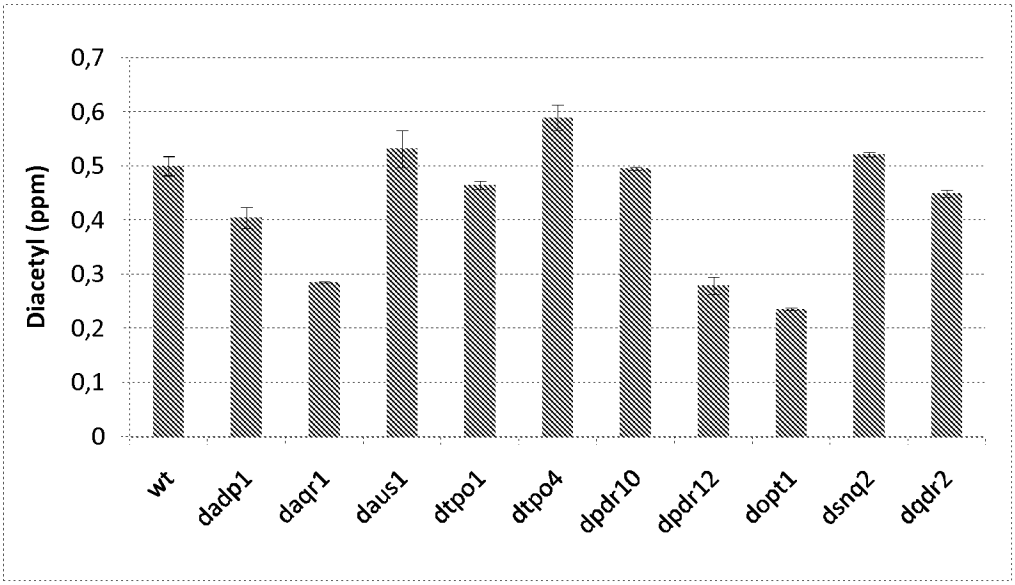


Figure 2

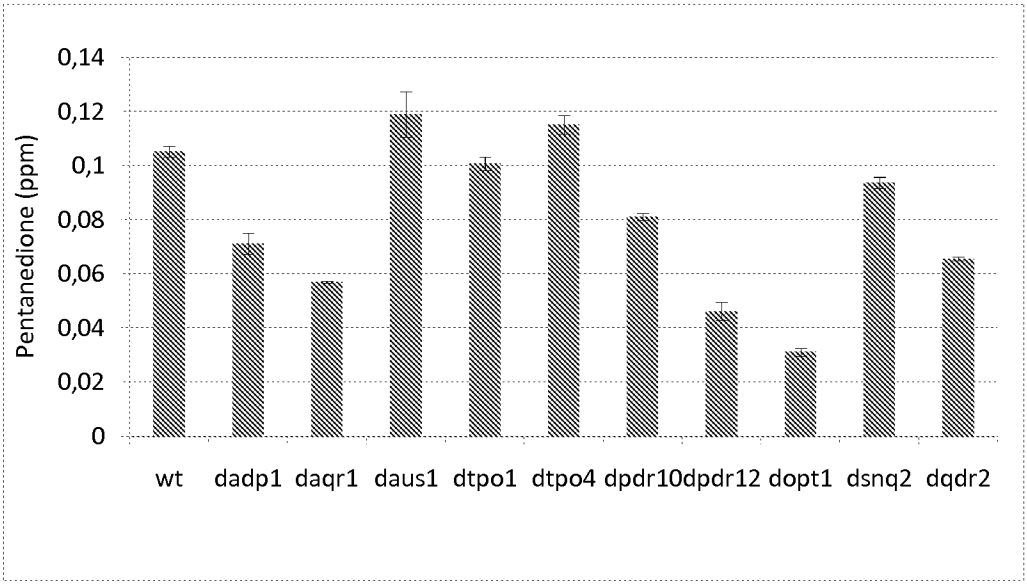


Figure 3

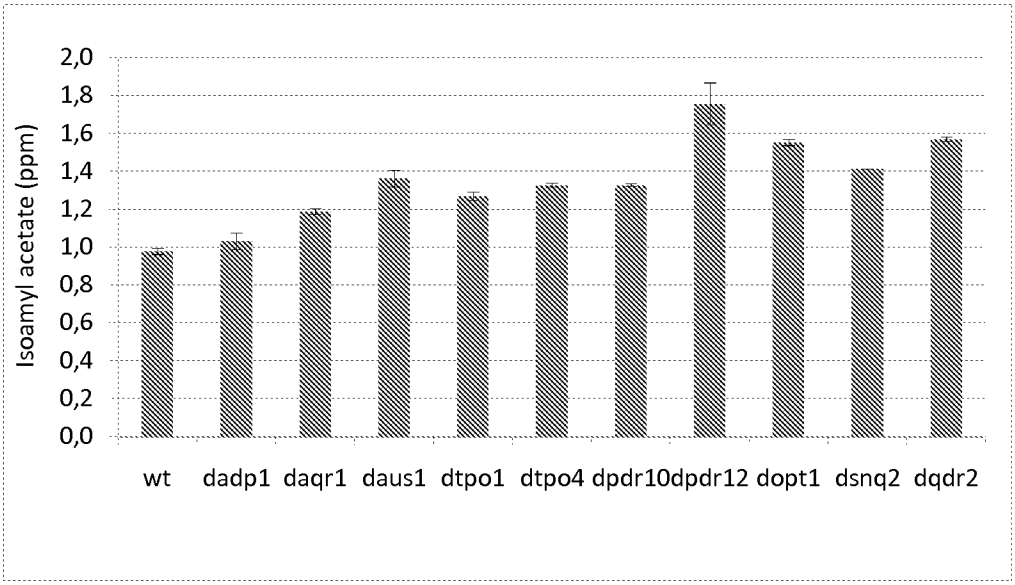


Figure 4

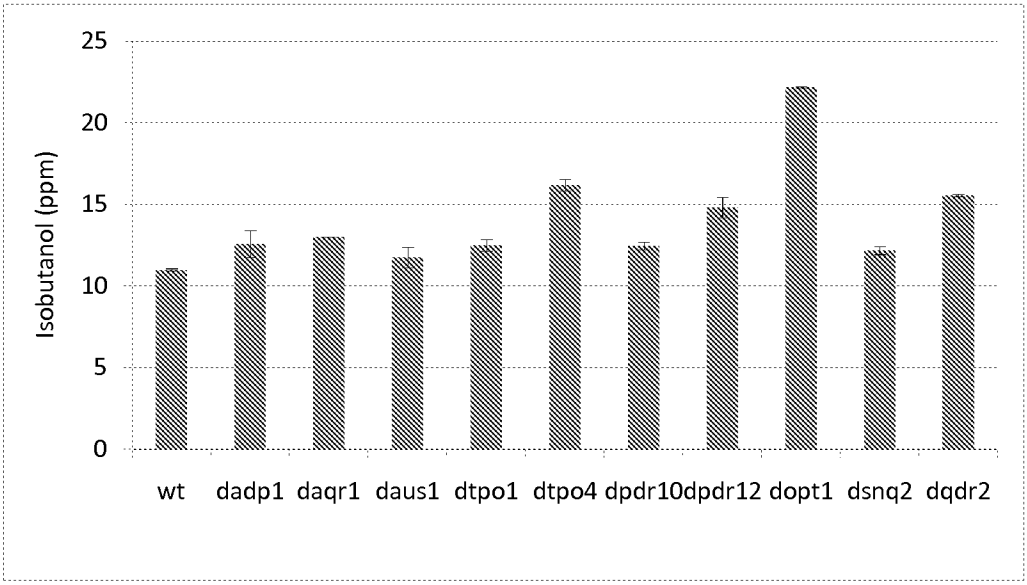


Figure 5

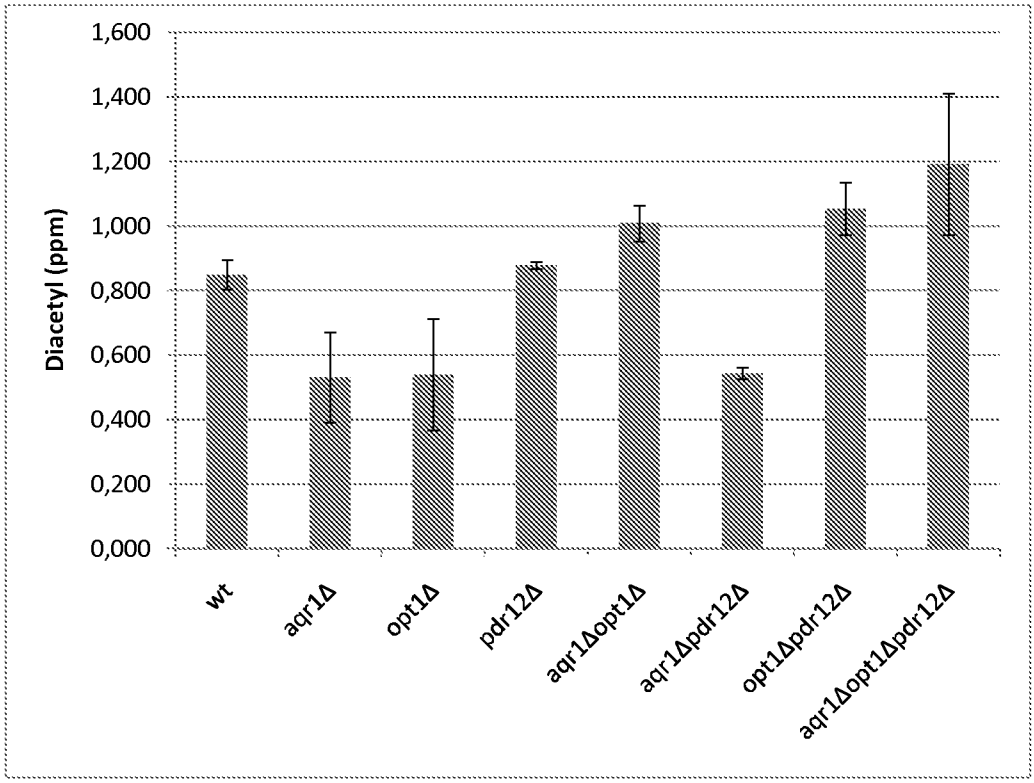


Figure 6

