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ORIGINAL RESEARCH ARTICLE

Genome sequencing and oenologically relevant traits of the Uruguayan native yeast *Issatchenkia terricola*

Juliette Dourron[1], Stefani de Ovalle[2], Paula González-Pombo[2], Andrea Villarino[1], Ana Ramón[1]* and Alicia Costábile[1]*

[1] Sección Bioquímica, Departamento de Biología Celular y Molecular, Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400, Montevideo, Uruguay
[2] Área Bioquímica, Departamento de Biociencias, Facultad de Química, Universidad de la República, Gral. Flores 2124, CC1157 Montevideo, Uruguay

*correspondence:
anaramon@fcien.edu.uy
acostabile@fcien.edu.uy

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ABSTRACT

Issatchenkia terricola 0621 is a non-*Saccharomyces* yeast strain isolated from Tannat grapes from Uruguayan vineyards; it stands out for its ability to produce high levels of β-glucosidase activity, which contributes to the aromatic complexity of wines. To delve into the potential oenological applications of this strain, its high-quality genome was obtained and explored, allowing the main central carbon and nitrogen metabolic pathways to be reconstructed. *I. terricola* is able to utilise glycerol as the sole carbon source in a way that has not previously been described for yeasts. The genes of the fermentome and those involved in stress resistance during winemaking were also identified, and differences were found when compared to *S. cerevisiae*, which may explain why *I. terricola* is unable to complete fermentation. The pathways responsible for natural aroma synthesis were also reconstructed, and the production of aromatic acids, alcohols, esters, acetates and lactones was verified experimentally. Finally, sequences encoding for β-glucosidases, in addition to the previously characterised one, were identified in the genome. The work presented here lays the groundwork for experimental research focused on the dissection of the metabolism of a native non-*Saccharomyces* strain and its application for oenological and biotechnological purposes.

KEYWORDS: Genome, non-*Saccharomyces*, *Issatchenkia terricola*, metabolism, wine

INTRODUCTION

In wine production, yeast strains not only have a leading role in the alcoholic fermentation process but they are also responsible for the final characteristics of the beverage, such as colour, structure and aromatic composition (Lambrechts and Pretorius, 2000). *Saccharomyces cerevisiae* is considered the top fermenting yeast, but non-Saccharomyces (NS) yeasts that survive the early stages of alcoholic fermentation, and that represent 50–70 % of the total wine yeast population, are attracting increasing attention due to their different abilities to secrete enzymes, produce secondary metabolites, glycerol and ethanol and release mannoproteins, among others (de Ovalle *et al*., 2021; Jolly *et al*., 2006). Thus, an adequate selection and use of NS strains may contribute to the organoleptic and sensory properties of the final product when they are used in mixed fermentations with *S. cerevisiae* (Shi *et al*., 2019). More than fifteen NS yeast genera associated with wine fermentation have been identified so far, and, in the last decade, research on NS strains has advanced notably, with a focus on the improvement of specific parameters of wine quality and its positive role in the organoleptic characteristics of wine (de Ovalle *et al*., 2018; Padilla *et al*., 2016).

With a mild Atlantic climate comparable to that of European wine regions, Uruguay is the fourth largest wine producer in South America. In the last few years, Uruguay has focused research on native strains and their use in wine fermentations (de Ovalle *et al*., 2021; Lleixà *et al*., 2016). In previous research, native yeast producers of β-glucosidase (BGL) activity, *Metschnikowia pulcherrima*, *Issatchenkia terricola* and *Issatchenkia orientalis* were isolated from Uruguayan grapes and vineyards (de Ovalle *et al*., 2018, 2021; González-Pombo *et al*., 2008, 2011). These enzymes play a crucial role in releasing volatile compounds from non-volatile glycosidic precursors present in must and wines. In the case of *I. terricola,* we found that BGL activity stands out and increases the level of free monoterpenes and norisoprenoids, which contribute to the aromatic complexity of Muscat and Tannat wines (de Ovalle *et al*., 2018; González-Pombo *et al*., 2011). The ability of strains of this species to produce high levels of BGL has also been reported by other authors, who also highlighted their potential for reducing the total acidity content of wines and producing acetaldehyde (Shi *et al*., 2019; W. Zhang *et al*., 2020).

Having knowledge of the genomes of NS yeasts is expected to contribute considerably to the understanding of their metabolic and ecological diversity, which is essential for their successful biotechnological application (Seixas *et al*., 2019). Nevertheless, sequenced and characterised genomes of native NS strains are still very scarce. Hence, to provide a basis for further research on the use of *I. terricola* in oenology, in this study we carried out the sequencing, annotation and functional analysis of the genome of strain 0621 isolated from Tannat grapes of a Uruguayan vineyard. In the study, there is an emphasis on the metabolic traits relevant to winemaking and the strain´s tolerance to the many stress factors that arise throughout the fermentation process.

MATERIALS AND METHODS

1. *I. terricola* culture conditions

Issatchenkia terricola 0621 isolated from Tannat grapes (González-Pombo *et al*., 2011) was precultured on a YPD medium (0.1 g/L yeast extract, 2 g/L peptone, 2 g/L glucose) and then cultured in simile must (Martin *et al*., 2016) or synthetic mediums (de Ovalle *et al*., 2018) at 28 °C and 100–180 rpm. For the growth assays, the required amount of preculture to reach an initial OD_{600} of 0.2 was inoculated in 50 mL of synthetic medium. The latter was prepared with no carbon or nitrogen sources, which were added after sterilisation. The carbon sources comprised: glucose (10 g/L), fructose (10 g/L) or glycerol (8 mL/L). The nitrogen sources comprised: urea (4 g/L), ammonium (140 or 200 mg/L) or L-amino acids (Pro 20.5 mg/L, Gln 16.9 mg/L, Arg 1.25 mg/L, Trp 6.0 mg/L, Ala 4.9 mg/L, Glu 4.0 mg/L, Ser 2.6 mg/L, Thr 2.6 mg/L, Leu 1.6 mg/L, Asp 1.5 mg/L, Val 1.5 mg/L, Phe 1.3 mg/L, Ile 1.1 mg/L, His 1.1 mg/L, Met 1.1 mg/L, Tyr 0.6 mg/L, Gly 0.6 mg/L, Lys 0.6 mg/L, Cys 0.4 mg/L). The OD_{600} of the culture was monitored every day until no more growth was observed.

Fermentations were assayed on the must from Viura grapes, which were pressed and then pasteurised with a short cycle of temperature increase to 105 °C and subsequent gradual decrease. Assays were performed in conical bottom tubes containing 40 mL of the must at 1.06 g/mL and containing 222 g/L of glucose and fructose. Each tube was inoculated with a preculture of *I. terricola* to an initial OD_{600} of 0.2. Culture conditions were initially aerobic (180 rpm, 28 °C) for 3 days, and then they were anaerobic for 8 days (with airlock valves). Fermentation was monitored by weight loss.

2. Analytical identification of volatile compounds

The volatile fraction was obtained by headspace solid-phase micro extraction coupled with gas chromatography-mass spectrometry (HS-SPME/GCMS), as previously described (Pozo-Bayón *et al*., 2001). A Trace GC Ultra gas chromatograph with a Triplus Autosampler and ISQ mass detector was used (Thermo Scientific). In a 20 mL headspace-vial, 4 g of NaCl was added to 2 mL of wine sample, followed by 40 µL of internal standard solution (1000 ppm 4-methyl-2-pentanol, 20 ppm nonanol, and 1000 ppm n-heptanoic, in ethanol), which was filled up to a final volume of 9 mL with distilled water. The vial was tightly capped with a PTFE/Silicone cap and heated at 70 °C for 10 min. Then, a Supelco 100 µm PDMS fibre was exposed to the headspace of the sample vials for 30 min, after which volatiles were desorbed in the GC inlet for 4 min. The instrument was fitted with a 30 m × 0.25 mm TG-WAXMS fused-silica capillary column, 0.25 mm film thickness (Thermo, Fisher Scientific). The GC temperature programme was 40 °C (5 min hold), 3 °C/min up to 200 °C and 15 °C/min^{-1} up to 240 °C (10 min hold),

while the SPME liner (0.75 mm ID) was held at 180 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min, operating in split mode (ratio 30). For the MS detector, the temperature of both the transfer line and ion source was 250 °C, the ionisation mode was electron impact at a voltage of 70 eV, and acquisitions were performed in SIM mode (dwell time 50 ms). Instrument control, data analysis and quantification results were carried out with the Xcalibur 2.1 software. Volatile compounds were identified and quantified as duplicates by comparison with standards and expressed as the value of the area ratio (sample/standard).

3. DNA and RNA extraction and sequencing

200 mL of YPD was inoculated with a preculture of *I. terricola* to an initial OD_{600} of 0.2 and growth monitored for 6 days. DNA was extracted using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue N°: D6005). mRNA was extracted from yeast cultures grown on simile must (Martin *et al*., 2016) or on synthetic medium (de Ovalle *et al*., 2018). Total RNA was extracted using Quick-RNA™ Miniprep Kit (Zymo Research, Catalogue N°: R1054). To improve the quality and quantity of both DNA and mRNA, an additional step of cell disruption (2 min in a Mini-Beadbeater-16, Biospec products) was performed after the addition of Lysis Buffer.

DNA was sequenced with both Illumina and PacBio technologies. Libraries were constructed and sequenced in Macrogen (Korea). Illumina libraries were constructed using TruSeq DNA Sample Prep Kit (Illumina) and sequenced in Hiseq2500 equipment producing 100 bp paired end reads. A SMRTbell 20kb library was constructed and sequenced in one PacBio RSII SMRT cell. mRNA of *I. terricola* was sequenced using Illumina technology. Libraries were constructed using TruSeq mRNA library after polyA RNA purification and sequenced with Hiseq2500, producing 100 bp paired end reads.

4. Genome assembly and annotation

The quality of Illumina reads was analysed with FastQC (v0.11.5, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Bases with Phred score lower than 30, as well as the first ten bases of all reads, were trimmed using Trimmomatic (v0.36, Bolger *et al*., 2014). PacBio reads were corrected using Illumina reads with LoRDEC (v0.9, Salmela and Rivals, 2014) with parameters k: 19 s: 3. Genome assembly was done based on Illumina-corrected long reads using Flye (v2.3.6, Kolmogorov *et al*., 2019) with default parameters and genome size of 12Mb. For assembly polishing, short reads were mapped to the obtained genome assembly using Bowtie2 (v2.3.4.2, Langmead and Salzberg, 2012) with default parameters, and used as input to Pilon (v1.22, Walker *et al*., 2014a). The quality of the assembly was evaluated using Quast (v5.0.0, Gurevich *et al*., 2013), with the options "conserved genes finding" and "fungus". Genome completeness was assessed using BUSCO (v3.0.2, Waterhouse *et al*., 2018) against *ascomycete* lineage downloaded from OrthoDB (v9.1, Zdobnov *et al*., 2017) using default parameters. Mitochondrial genome assembly was performed using Norgal (v0.1, Al-Nakeeb *et al*., 2017) with default parameters, using the short reads obtained with Illumina as input. BLASTn (v2.6.0+, Camacho *et al*., 2009) was used to determine homology between Norgal mitochondrial and Flye total genome assemblies with the parameter "e-value 1e-5".

For gene annotation, Braker (v1.9, Hoff et al., 2016) was used, with the *fungus* option and all other parameters set in default. The mapping of RNAseq reads against the assembled genome was done with HISAT2 using default parameters (v2.0.0-beta, Kim *et al*., 2015). The mapping file was used to train GeneMark and AUGUSTUS gene predictors. Annotation quality was assessed with BUSCO (v3.0.2, Waterhouse *et al*., 2018) with annotated proteins as entry (-m prot parameter) against the *ascomycete* linage from the OrthoDB database (v9.1, Zdobnov *et al*., 2017), with default parameters. Mitochondrial annotation was performed using Mitos Web Server (Donath *et al*., 2019), using Yeast genetic code and RefSeq 63 Fungi as the reference.

5. Functional annotation

InterProScan (v5.31.70.0, Jones et al., 2014) was used for protein domain assignment, with the option "goterm" to report Gene Ontology terms (GO) and the remaining parameters as default. A BLASTp search (v2.6.0+, Camacho *et al*., 2009) was run against the Swiss-Prot database (downloaded in February 2019, Poux *et al*., 2017) using the *I. terricola* annotated proteins as query, and "e-value 1e-5", "max_target_seqs 1" and the other options set in default. KEGG terms were assigned with the online KAAS tool (Moriya *et al*., 2007), using the GHOSTX search program, *Saccharomycetes* organisms as Gene datasets, and BBH (bi-directional best hit) as assignment method. KEGG Pathways assignment and Brite classification were performed with the KEGG Reconstruct tool (Kanehisa and Sato, 2020). For comparison, the same analysis was performed for *S. cerevisiae* and *Pichia kudriavzevii.*

Protein sequences from related yeasts with available annotated genomes were downloaded (see Supplementary Table 1). Orthologs were determined using Orthofinder (v2.4.0, Emms and Kelly, 2019) with options M: msa and S: diamond. *S. cerevisiae* genes of interest were used to retrieve *I. terricola* orthologous genes from orthologous cluster analysis. When no clear ortholog gene could be determined, Enzyme Commission numbers or protein domains (Pfam or InterPro) of *S. cerevisiae* proteins were obtained from UniProt and searched in *I. terricola* annotation. *S. cerevisiae* proteins were also used as queries for local BLASTp or BLASTx (v2.6.0+, Camacho *et al*., 2009) using the *I. terricola* proteome and genome as databases respectively. For these, an e-value cut-off of 1e-5 was used and the BLAST results were manually filtered for each protein.

6. Identification of β-glucosidases in the genome of *I. terricola*

Different methods were used for the identification of β-glucosidases in *I. terricola*. First, enzymes with the assigned term "beta-glucosidase" from InterProScan

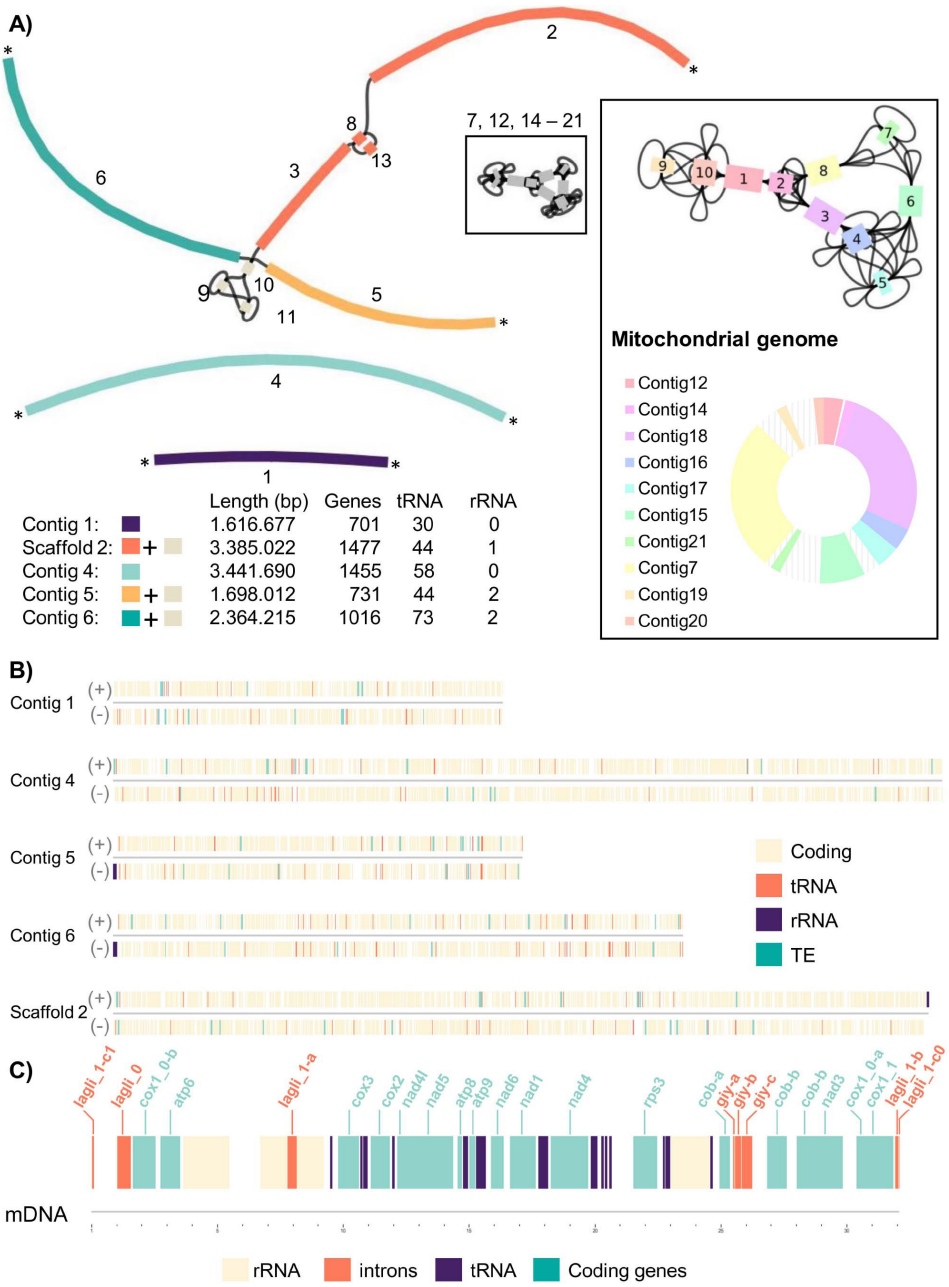

FIGURE 1. *I. terricola* genome assembly and annotation.

(A): Genome assembly graph visualized with Bandage (v0.8.1, Wick *et al.*, 2015). Contigs and scaffold were assembled using Flye and polished with Pilon. Coloured segments correspond to chromosomes. Three short segments (light brown squares) corresponding to rRNA sequences were included in Scaffold 2, Contigs 5 and 6. Putative telomeres are indicated by asterisks (*). Segment lengths and the number of annotated genes are shown. The grey segments enclosed in the small inset correspond to mitochondrial fragments assembled by Flye. The order of the different segments is shown in the big inset, as obtained by comparison with the mitochondrial genome. (B): Annotation of the *I. terricola* nuclear genome. Coding genes (yellow), tRNAs (orange), rRNA clusters (purple) and transposable elements (TE) (aquamarine) are shown in each nuclear contig. (C): Mitochondrial genome annotation. Coding genes for NADH:ubiquinone oxidoreductase subunits (nad), the 3 subunits of ATP synthase (atp), the 3 subunits of the cytochrome c oxidase (cox), apocytochrome b (cob-a), and ribosomal protein S3 (rps3) are shown in aquamarine, tRNAs are shown in purple, rRNAs are shown in yellow and mobile introns (LAGLI, GIY) in genes cox1 and cob are indicated in orange. Plots in B and C were obtained using the karyoploteR package (v1.16.0, Gel and Serra, 2017).

analysis and homology to Swiss-Prot β-glucosidases were retrieved from genome functional annotation. Second, BLAST homology search of annotated proteins with those from existing databases was performed. The BLASTp programme (v2.6.0+, Camacho *et al.*, 2009) was used with "e-value 1e-5", "max_target_seq 1" and the other parameters by default. Yeast β-glucosidases were retrieved from NCBI Refseq protein database (research performed 26th February 2022), searching by the terms "beta glucosidase" in all fields, with the filter "Organism = fungi and budding yeasts". These protein sequences were used for BLASTp search against *I. terricola* annotated proteins. The dbCAN2

meta server (H. Zhang *et al*., 2018) was used to classify the identified glucosidases using the three available tools DIAMOND, HMMER y Hotpep.

RESULTS

1. Genome assembly

To obtain the genome of the *I. terricola* 0621 reference strain, we followed a hybrid strategy combining PacBio long reads and Illumina short reads. Genome sequencing produced 138.868 long reads and 22.509.739 short, paired end reads. The total genome assembly size was 12.5 Mb, consisting of 15 scaffolds, 5 of them longer than 1Mb (Figure 1A), with an N_{50} of 3,4 Mb and a GC content of 36.4 %, similar to those of related yeasts (Douglass *et al*., 2018). The mitochondrial genome assembly, obtained exclusively from short reads, produced a circular scaffold of 32.088 bp, showing high homology to the shorter contigs of the assembly obtained from the long reads (contigs 7, 12, 14-21, Figure 1A). These results suggest that the shorter contigs are fragments of the mitochondrial genome, while longer scaffolds correspond to nuclear chromosomes. Genome polishing resulted in the correction of 137 single nucleotide polymorphisms (SNPs), 1793 small insertions (making up a total of 6,911 bases), and 119 small deletions (making up a total of 195 bases). No ambiguous bases were found. The polishing result did not give major differences in the quality metrics with respect to the originally assembled genome (Supplementary Table 2). The coverage was 76X and 25729X for the nuclear and mitochondrial genomes, respectively. Raw reads and genome assembly were deposited in NCBI under Bioproject PRJNA833333.

2. Genome annotation

5299 coding genes were annotated in the *I. terricola* nuclear genome, corresponding to 5377 open reading frames (ORFs) of putative proteins. Most of the genes coded for one isoform, while 132 coded for two or more isoforms. Of these 5299 genes, 98 were annotated as transposable elements (Figure 1B and Supplementary Table 3).

A total of 293 tRNA genes were annotated and distributed in all contigs. Three clusters of rRNA genes are localised in the extremes of Contig 5, Contig 6, and Scaffold 2. The latter include genes for the Large Subunit (LSU), 5.8S, the Small Subunit (SSU) and 5S. Due to their repetitive nature, the ends of these fragments could not be resolved (Figure 1A). Moreover, several other non-coding genes were annotated (Supplementary Table 4).

The mitochondrial genome coded for 15 proteins, 25 tRNAs and the small and large subunits of rRNA (Figure 1C and Supplementary Table 5).

Genome completeness analysis revealed that of the 1315 genes conserved in all the Ascomycetes, 92.3 % were complete, 3.1 % were fragmented and 4.6 % were missing in *I. terricola* (Table 1). These metrics are similar to those obtained for the related yeast *P. kudriavzevii* (Douglass *et al*., 2018). Indeed, the number of genes per chromosome obtained in the assembly of each of the two species is also similar (Table 1).

Of the 5377 annotated proteins, 97 % (5216) have domains predicted by InterProScan: 87 % (4676) have an InterPro domain, 81 % (4379) have a Pfam domain, and 82 % (4417) were assigned a PANTHER database entry. Signal peptides were identified in 6.5 % (349) of the proteins, and 18.6 % (1000 proteins) showed transmembrane regions. A GO term was assigned to 67.8 % (3,644) of the proteins (Figure 2A and Supplementary Table 3).

To determine the number of genes that have homologs in other organisms, a BLASTp search against the Swiss-Prot database of curated proteins was performed. Of all *I. terricola* annotated proteins, 84,2 % (4527) aligned with at least one

TABLE 1. Statistics for *I. terricola* nuclear genome and comparison to *P. kudriavzevii* genome.

		I. terricola		*P. kudriavzevii* (GCA_00305445)	
General Metrics	Contigs	5		5	
	Genes	5299 coding		5140 coding	
		300 non-coding		226 non-coding	
	CDS	5377		5154	
		Length (Mb)	Genes	Length (Mb)	Genes
Genes per contig	Chromosome 1	3.442	1423	2.852	1346
	Chromosome 2	3.385	1426	2.746	1322
	Chromosome 3	2.364	995	2.542	1204
	Chromosome 4	1.698	712	1.384	670
	Chromosome 5	1.617	673	1.289	598
BUSCO Analysis (Ascomycete lineage)	Complete	92.3 %		91.7 %	
	Fragmented	3.1 %		4.3 %	
	Missing	4.6 %		4.0 %	
	Total	1315			

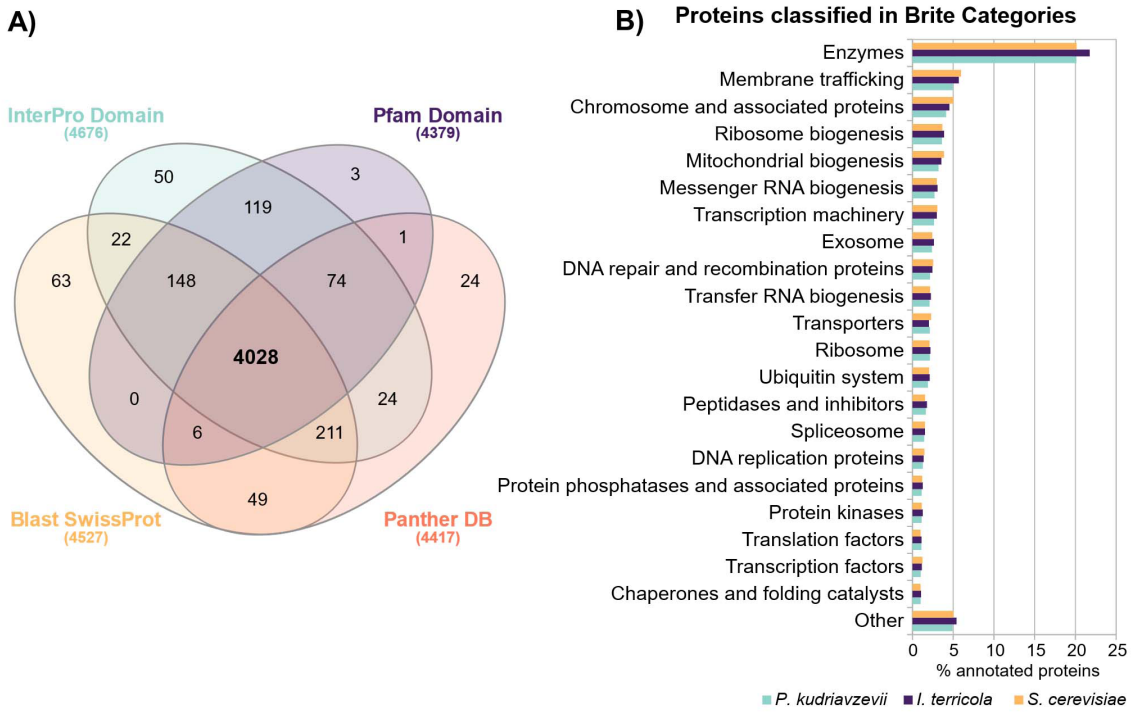

FIGURE 2. Functional annotation of predicted proteins.

(A): Venn Diagram for InterProScan and BLAST against Swiss-Prot results obtained with InteractiVenn (Heberle *et al.*, 2015). Of the 5377 proteins, 4676 were annotated with an InterPro domain (green), 4379 with a Pfam domain (purple), 4417 with a Panther Database entry (pink), and 4527 show homology with curated proteins (Swiss-Prot database, orange). 4028 proteins meet the four conditions. The number of proteins annotated using each of these methods or their intersection is shown in each sector of the diagram. (B): Comparison of proteins assigned to Brite categories in *I. terricola* (purple), *P. kudriavzevii* (light blue) and *S. cerevisiae* (light orange).

protein of the database, of which 4228 were fungal proteins (Figure 2A and Supplementary Table 3).

A KEGG pathway analysis allowed the metabolic routes present in *I. terricola* to be identified and their completeness to be assessed. Of the 5377 proteins predicted in *I. terricola*, 2834 (51 %) were assigned to KEGG orthology terms and classified according to the Brite hierarchy. The analysis was performed in parallel for the related organisms *S. cerevisiae* and *P. kudriavzevii* as a reference (Figure 2B).

The predicted proteins were used for the identification of the metabolic pathways involved in the utilisation of carbon and nitrogen sources present in wine musts, as well as those involved in stress management, or aroma and flavour production. In some cases, experiments were done to support the identification of the metabolic processes.

3. Carbon and nitrogen metabolism

We looked for and identified the enzymes of *I. terricola* involved in the main central carbon metabolism (glycolysis, TCA cycle and pentose phosphate pathway, as well as in gluconeogenesis) (Supplementary Figure 1). We also searched for the genes of the "fermentome" of *I. terricola*, based on the data available for *S. cerevisiae* growing on synthetic grape juice (Walker *et al.*, 2014b). Of the 90 genes composing this fermentome, 78 were retrieved in *I. terricola* (Supplementary Table 6). Moreover, our results show that homologs of several of the genes that in *S. cerevisiae* have been found to be essential for fermentation (*RBL2*, *ZAP1*, *YFL012*, *VMA22*) or determinant for its duration (*MEH1* and *SLM4*, of the EGO/GSE complex and *VAM3*, associated with vacuolar acidification), are absent in *I. terricola* (Walker *et al.*, 2014b).

Since grape musts are rich in hexoses other than glucose (fructose, galactose and mannose), pentoses (arabinose, xylose and ribose), lactate, acetate and lipids that yeasts can utilise as carbon sources (Liu and Davis, 1994; Muñoz *et al.*, 2011), we also looked for the enzymes involved in the catabolism of these compounds. We were able to find in the genome of *I. terricola* the complete utilisation pathways for all the above-mentioned carbon sources, except for galactose (Table 2).

During must fermentation, *S. cerevisiae* produces glycerol as a by-product, which can then be utilised by other yeasts as a carbon source with varying efficiency (Scanes *et al.*, 1998). Three different glycerol catabolic pathways have been reported in yeast and other fungi (Figure 3A): 1) the catabolic G3P pathway, 2) the catabolic DHA pathway, and 3) the catabolic D-glyceraldehyde (GA) pathway, which is then converted to

TABLE 2. *I. terricola* genes involved in the catabolism of carbon sources other than glucose.

Carbon source	Enzyme	*I. terricola* gene
Fructose	Hexokinase	g4909.t1
	6-phosphofructokinase	g1705.t1, g3386.t1
	Fructose-bisphosphate aldolase	g1321.t1
Mannose	Hexokinase	g2005.t1, g2787.t1, g4909.t1
	Mannose 6P isomerase	g4613.t1
Arabinose	D-arabinitol 2-dehydrogenase	g2437.t1
Xylose	D-xylose reductase	g584.t1
	Xylitol dehydrogenase	g4327.t1
	Xylulose kinase	g.4330.t1
	Ribulose phosphate 3-epimerase	g1689.t1
Glycerol	Glycerol dehydrogenase	g3745.t1
	Dihydroxyacetone kinase	g1476.t1
	Aldehyde dehydrogenase	g1114.t1, g1837.t1, g3795.t1
	Glycerate 3-kinase	g2930.t1
Lactate	D-Lactate dehydrogenase	g577.t1; g5228.t1
	L-Lactate dehydrogenase	g3765.t1, g4470.t1, g 4946.t1
Acetate	Acetyl-coenzyme A synthetase	g4173.t1, g4279.t1
	Fatty-acyl coenzyme A oxidase	g4331.t1
Lipids	3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase	g1315.t1
	3-ketoacyl-CoA thiolase	g2686.t1
	Triacylglycerol and Monoglyceride lipases	g1726.t1, g817.t1, g524.t1, g3700.t1, g1944.t1

i) glyceraldehyde 3-phosphate or ii) 3-phospho D-glycerate to enter glycolysis or gluconeogenesis (Klein *et al*., 2017). We identified in *I. terricola* the enzymes of the GA and the DHA routes (Figure 3A). However, whether the identified ortholog of *S. cerevisiae* GCY1/YPR1 catalyses the first step in the route remains to be determined, since in the budding yeast none of these genes showed NAD^+-dependent activity. The ability of *I. terricola* to use glycerol as the sole carbon source was experimentally demonstrated by performing growth curves (Figure 3B), with ammonium or amino acids as nitrogen sources. On glycerol + ammonium, the cultures showed an exponential growth for the first 24 hours, after which the culture reached the stationary phase. On amino acids as nitrogen source the exponential growth continued for almost 50 hours, when it started to slow down.

Another source of glycerol is its mobilisation from stored lipids, such as triacylglycerols (TAGs). In this pathway, TAGs are transformed into diacylglycerols (DG), which are then converted into monoacylglycerols (MG), to finally release glycerol and free fatty acids, which constitute a relevant energy source (Klug and Daum, 2014). The analysis of the *I. terricola* genome showed that this yeast has all the genes corresponding to enzymes involved in the fatty acid metabolism (Figure 4 and Supplementary Table 7). When required by the cells, free fatty acids can be derived from stored lipids, as well as from *de novo* synthesis or uptake of external lipids, and they can be used as a source of energy and for the synthesis of membrane lipids, or stored in organelles, such as lipid droplets (Tesnière, 2019).

Concerning nitrogen sources, essential for biomass production and efficient fermentation, a wide variety of compounds assimilable by yeasts are also present in grape musts. Among them, amino acids or ammonium are generally the primary ones (Santamaría *et al*., 2020), but there are also minor sources like urea, small peptides and ornithine, etc. As expected, the genes that ensure the assimilation of ammonium in glutamate and glutamine were identified in *I. terricola*. In addition, pathways for all amino acid synthesis except Cys were identified, as well as those involved in the catabolism of some of them (Supplementary Figure 1).

4. *I. terricola* transporters

More than 500 genes in *I. terricola* were predicted to encode transporters of different substances across the cell membranes, regulators of this function or sensors (Supplementary Table 8). Among these, and given the complex and variable composition of nutrient availability in natural growth substrates of *I. terricola* and in musts during winemaking, it was not surprising to find a set of proteins that are able to transport a huge variety of compounds across the membranes (Supplementary Table 8). We identified 17 genes that encode transporters with putative specificity for different carbon sources (hexoses, carboxylic acids, and glycerol) and five genes coding for proteins similar to different yeast hexose transporters. We also found ten proteins predicted

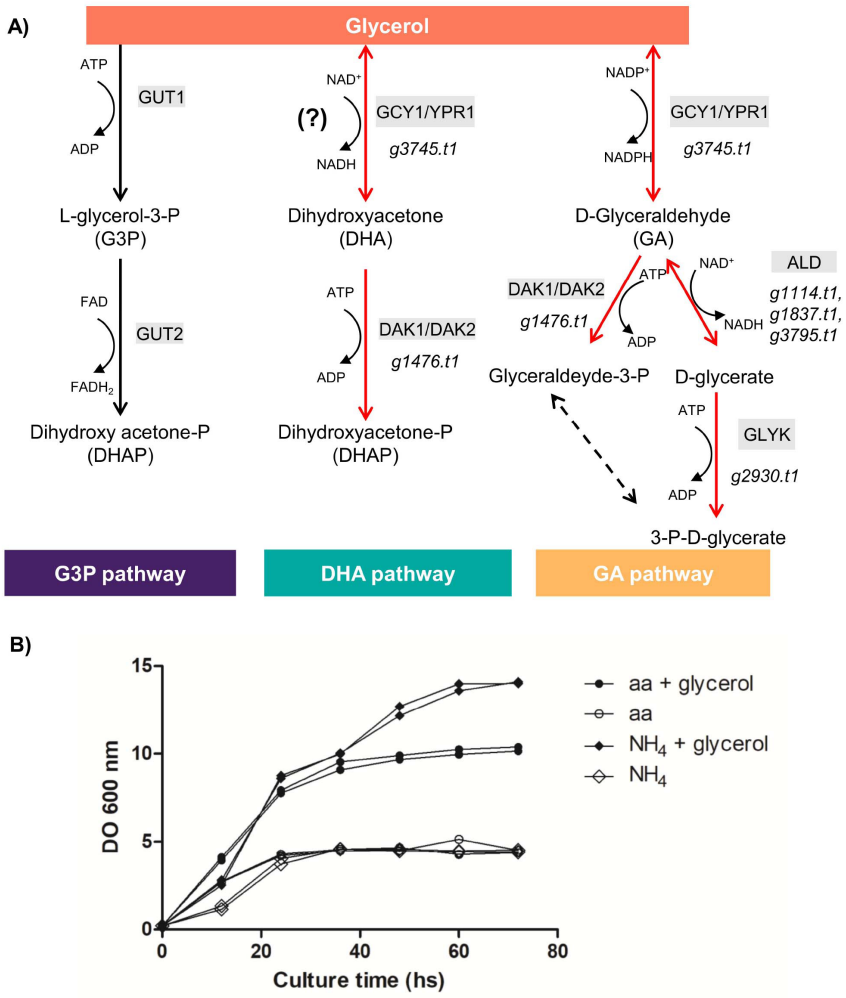

FIGURE 3. *I. terricola* is able to grow on glycerol as sole carbon source.

(A): Different suggested glycerol catabolism pathways in fungi (Klein *et al.*, 2017), and those putatively present in *I. terricola*: 1) the G3P pathway; 2) the DHA pathway, and 3) the D-glyceraldehyde (GA) pathway. The final products of the three pathways then enter glycolysis or gluconeogenesis. GUT1: glycerol kinase (E.C. 2.7.1.30); GUT2: FAD-dependent glycerol 3-phosphate dehydrogenase (E.C. 1.1.5.3); DAK1/DAK2: Dihydroxyacetone kinase (E.C. 2.7.1.29); GCY/YPR1: NAD^+/$NADP^+$dependent glycerol dehydrogenase (E.C. 1.1.1.72); ALD: aldehyde dehydrogenase (E.C. 1.2.1.3); GLYK: glycerate 3-kinase (E.C. 2.7.1.31). When present, genes encoding *I. terricola* homologs of the enzymes are indicated in italics next to the enzyme name, and the steps they catalyse are indicated by red arrows. (?): NAD^+dependent glycerol dehydrogenase activity needs confirmation (B): Growth curves on glycerol (gly) as sole carbon source, using amino acids (aa) or ammonium (NH_4) as nitrogen sources. The optical density at 600 nm was plotted against the culture time, measured in hours. The standard deviation was less than 10 %.

as carboxylic acids transporters, orthologs of *S. cerevisiae JEN1*, responsible for the high-affinity uptake of lactate and pyruvate or identified as homologs of the mammalian Mch family of monocarboxylates transporters. Even though no evidence of the Mch function has been found in yeast or other fungi, in *Aspergillus nidulans* the expression of a member of this group has been shown to be induced by acetate and pyruvate (Semighini *et al.*, 2004). Additionally, three genes are predicted to code for orthologs of STL1, GUP1 and GUP2 glycerol transporters.

As regards the uptake of nitrogenous compounds, the product of 40 genes show homology with membrane proteins with this function. Half of them bear similarities to fungal amino acid permeases, and especially to those with general specificity (9 out of the 20) or with transporters for specific amino acids (Met, Pro, Lys, Cys, Arg). The number of sequences having homology with the *S. cerevisiae* permease for the nonproteogenic amino acid gamma-aminobutyrate, GABA, also stands out (5 out of the 40 identified transporters for nitrogenous compounds). Permeases specific for ammonium, urea, purines, allantoin and allantoate were also identified. Furthermore, the genome of *I. terricola* carries several sequences recognised as transporters of different ions, vitamins, polyamines and choline, among others (Supplementary Table 8).

5. Genes relevant to coping with different stressors during wine production

In *S. cerevisiae,* the use of different high-throughput phenotypic screenings has allowed determinants of the

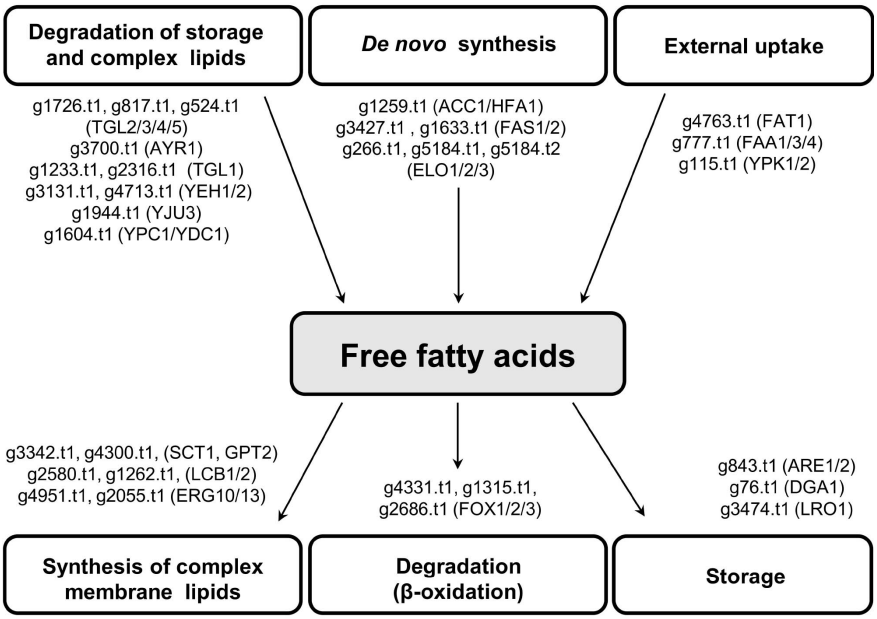

FIGURE 4. *I. terricola* genes corresponding to enzymes involved in fatty acid metabolism.

Free fatty acids are derived from the degradation of storage and complex lipids, *de novo* synthesis, and external uptake from the environment. Free fatty acid can be stored in TAGs and lipid droplets, incorporated into complex membrane lipids, or degraded by beta-oxidation. The name of the gene corresponding to *S. cerevisiae* is indicated in brackets. TGL2/3/4/5 and AYR1: triacylglycerol lipases (EC:3.1.1.3, EC:1.1.1.101), TGL1, YEH1/2: steryl ester hydrolases (EC:3.1.1.13), YJU3: monoglyceride lipase (EC:3.1.1.23), YPC1 and YDC1: ceramidases (EC:3.5.1.-), all involved in lipid mobilisation. ACC1 and HIF1: acetyl-CoA carboxylases (EC:6.4.1.2), FAS1/2: fatty acid synthetases (EC:2.3.1.86), ELO1/2/3: fatty acid elongases (EC:2.3.1.199). FAT1 and FAA1/3/4: fatty acyl-CoA synthetases and fatty acid transporters (EC:6.2.1.-, EC:6.2.1.3), YPK1/2: downstream serine/threonine protein kinase involved in sphingolipid-mediated signalling pathway (EC:2.7.11.1), ARE1/2, DGA1 and LRO1: acyltransferases in TAGs synthesis (EC:2.3.1.-); from β-oxidation: FOX1 fatty-acyl coenzyme A oxidase (EC:1.3.3.6), FOX2: 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase (EC4.2.1.119, EC:1.1.1.n12), FOX3: 3-ketoacyl-CoA thiolase (EC:2.3.1.16). SCT1 and GPT2: glycerol 3-phosphate/dihydroxyacetone phosphate sn-1 acyltransferases (EC:2.3.1.15, EC:2.3.1.42) in glycerolipid synthesis. LCB1/2: components of serine palmitoyltransferases (EC:2.3.1.50) in sphingolipid synthesis. ERG10 and 13: acetoacetyl-CoA thiolase (EC:2.3.1.9) and 3-hydroxy-3-methylglutaryl-CoA synthase (EC:2.3.1.10), respectively, involved in mevalonate biosynthesis. For more information see Supplementary Table 7.

resistance to the numerous stressors that occur during wine fermentation to be identified (Gonzalez *et al*., 2016; Teixeira *et al*., 2009; Teixeira *et al*., 2010). Based on these data, we analysed *I. terricola* for homologs of the genes involved in ethanol and high-sugar toxicity, which is of major importance (Supplementary Table 9). In the case of ethanol resistance, of the 641 identified genes in the budding yeast, 477 were found to be present in *I. terricola*. As in the case of *S. cerevisiae*, these genes are functionally related to vacuolar protein sorting and peroxisomal function, V-ATPase complex assembly, membrane and cell wall composition and the control of carbohydrate and amino acid metabolism. Regarding high-glucose tolerance, of the 278 genes identified as important or relevant in *S. cerevisiae*, 194 are present in *I. terricola* (Supplementary Table 9). Those genes are related to vacuolar organisation, late endosome to vacuole transport and the regulation of transcription, mainly at the level of nutrient metabolism control, as in the budding yeast (Gonzalez *et al*., 2016; Teixeira *et al*., 2010).

During alcoholic fermentation, adaptations in terms of lipid content are also important (Tesnière, 2019). *S. cerevisiae* imports unsaturated fatty acids (UFAs) and sterols through specific transporters or by endocytosis, to cope with the inability to synthesise these compounds in anaerobiosis (Tesnière, 2019). *I. terricola* have orthologous genes to *FAT1* and *YPK1* (g4763.t1 and g115.t1, respectively), possible regulators of fatty acid uptake in the budding yeast (Zou *et al*., 2002). Ethanol stress affects yeast membrane integrity, which is counteracted by an increase in fatty acid unsaturation (oleic acid), sterol and phosphatidylcholine content. In *I. terricola* we identified the genes and pathways involved in the synthesis of phospholipids, TAGs, sphingolipids and sterols as ergosterol, the most abundant lipids in yeast (Supplementary Table 7). In addition, during adaptation to the variable temperatures employed in winemaking, changes in membrane lipid content takes place (Henderson *et al*., 2013), eliciting the induction at the transcriptional level of genes involved in lipid biosynthetic pathways (López-Malo *et al*., 2014). It was possible to identify the *I. terricola* orthologs of these genes, such as DPL1 and LCB3 of the sphingolipid synthesis pathway (g512.t1 and g3137.t1 respectively), OLE1, which encodes for a desaturase (g4642.t1 and g5162.t1), or PSD1, which is involved in the synthesis of phosphatidylethanolamine (g4129.t1).

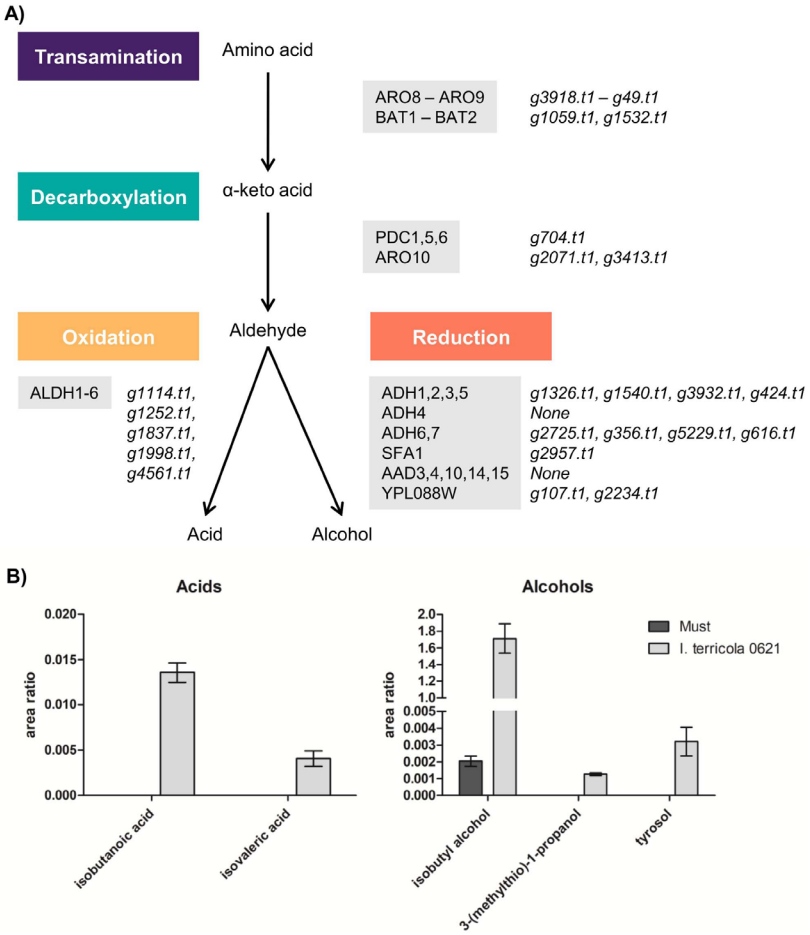

FIGURE 5. *I. terricola* genes related to aromatic compounds.

(A): *I. terricola* and *S. cerevisiae* genes encoding the enzymes involved in each of the steps of the Ehrlich pathway (transamination, decarboxylation and reduction or oxidation). BAT1 and BAT2: branched-chain aminotransferases (E.C. 2.6.1.42); ARO8 and ARO9 aromatic aminotransferases I and II (E.C. 2.6.1.57, E.C. 2.6.1.39); PDC1, 5 and 6: isoforms of pyruvate decarboxylase (E.C. 4.1.1.1); ARO10: phenylpyruvate decarboxylase (E.C. 4.1.1.-). ADH1-7: alcohol dehydrogenase (E.C. 1.1.1.1, E.C. 1.1.1.2); SFA1: bifunctional alcohol dehydrogenase and formaldehyde (E.C. 1.1.1.284); AAD3/4/10/14/15: aryl-alcohol dehydrogenase (E.C. 1.1.1.-), absent in *I. terricola*. ALDH1-6: aldehyde dehydrogenases (E.C. 1.2.1.3, E.C. 21.2.1.5). There are 5 ALDH genes in the *I. terricola* genome. YPL088W: Putative aryl alcohol dehydrogenase. When present, genes encoding *I. terricola* homologs of the enzymes are indicated in italics next to the enzyme name. (B): Acids and alcohols produced by *I. terricola* during Viura must fermentation, quantified by HS-SPME/GCMS as duplicates by comparison with standards and expressed as the value of the area ratio (sample/standard).

6. Natural flavours and fragrances

In the course of fermentation, maturation and aging, secondary metabolites present in musts are modified by yeasts and other microorganisms, resulting in an increase in the aroma complexity of wines. The compounds responsible for wine aroma are mainly alcohols, acids, esters, ketones and aldehydes, and their production depends on the set of microorganisms present in musts and the conditions applied during winemaking and aging (Ruiz *et al*., 2019).

6.1 Synthesis of aliphatic and aromatic alcohols (Ehrlich Pathway)

Wine fermentation is accompanied by the production of aliphatic and aromatic alcohols (fusel alcohols), which can contribute to the flavour and aroma of wine at low concentrations, but can cause off-flavours at high concentrations. These fusel alcohols are derived from the metabolism of valine, leucine, isoleucine, methionine and phenylalanine, which are taken up slowly throughout fermentation and enter the Ehrlich Pathway (Hazelwood *et al*., 2008). The reactions and enzyme-encoding genes involved in this pathway in *S. cerevisiae* and the identified counterparts in *I. terricola* are shown in Figure 5A. The ability of *I. terricola* to produce acids (isovaleric and isobutanoic), aliphatic alcohols (isobutyl alcohol, 3-methyl-thio-propanol) and the aromatic alcohol tyrosol during Viura must fermentation was experimentally observed (Figure 5B).

6.2 Synthesis of esters and other aroma compounds

During fermentations on Viura must, *I. terricola* was found to produce higher alcohol acetates or acetate esters, such as isobutyl acetate, and some fatty acid esters, such as ethyl lactate, ethyl tetradecanoate, ethyl oleate and ethyl

stearate (Figure 6C). These compounds are of particular interest, because they are responsible for the much desired fruity, candy and perfume-like aromas, being the most important set of yeast-derived aroma active compounds (Saerens *et al*., 2010). The pathways for the synthesis of these compounds and the participating *I. terricola* enzymes, identified by homology with their *S. cerevisiae* counterparts, are shown in Figure 6A-B.

6.3. Synthesis of carbonyl compounds

Yeasts are also able to synthesise many carbonyl compounds (aldehydes, ketones and keto acids) which can endow wines with fruity, nut-like, herbaceous and fatty notes, depending on the length of the alkyl group. Lactones are also formed in alcoholic fermentation (Ugliano and Henschke, 2009). We were able to determine experimentally that *I. terricola* is able to produce gamma-nonalactone (Figure 6D), which is considered to be the most important flavour of aged red wine (Ferreira, 2010) and which is associated with sweet and creamy notes (Lee and Noble, 2003). Hydroxyethyl gamma-butyrolactone is also produced by *I. terricola.* It is well known that gamma-C6 lactones are associated with red fruit aroma, such as strawberry, cherry and raspberry (Rocha *et al*., 2004). The hydroxyl fatty acid pathway (Krzyczkowska *et al*., 2017) accounts for most of the synthesis of lactones in yeast, and *I. terricola* was not the

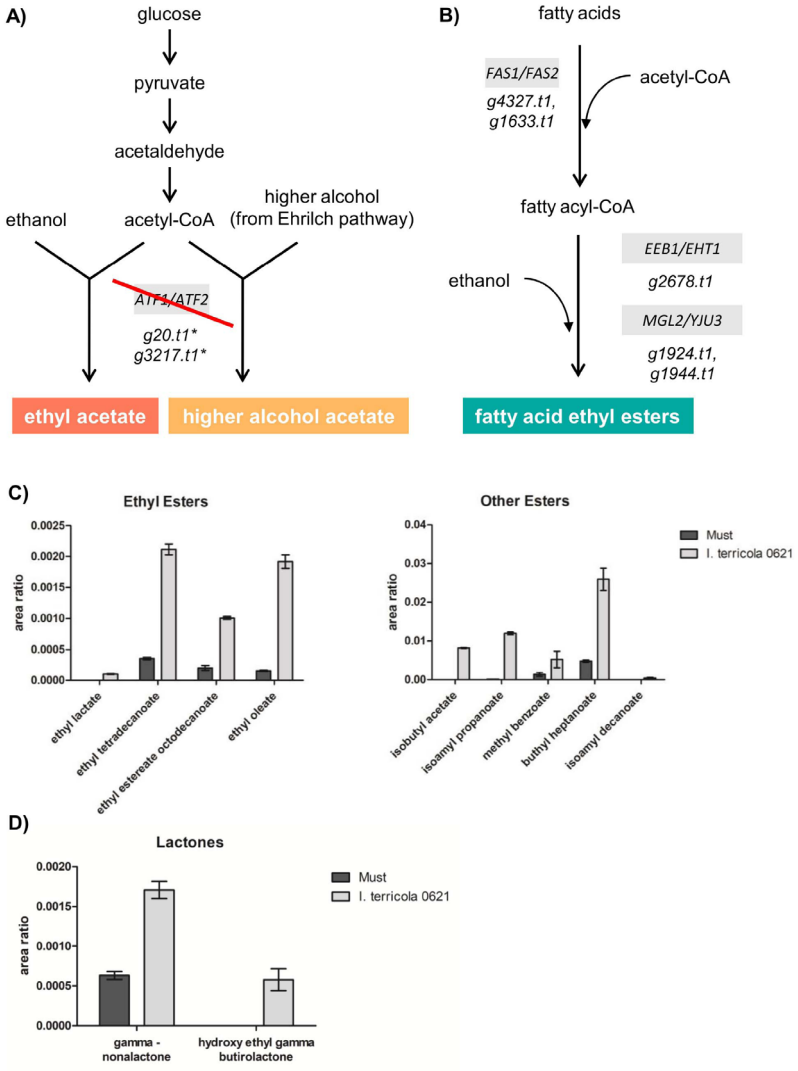

FIGURE 6. Synthesis of esters and carbonyl compounds in *I. terricola*.

(A): Ethanol or higher alcohols produced through the Ehrlich pathway react with acetyl-CoA to produce ethyl acetate or higher alcohol acetates respectively (Ugliano and Henschke, 2009). Orthologs of the *S. cerevisiae* genes involved, ATF1 and ATF2 (Ugliano and Henschke, 2009), are missing in *I. terricola*, but g20.t1 and/or g3217.t1, bearing an alcohol acetyltransferase/N-acetyltransferase domain (IPR10828), are good candidates for the fulfilment of this function (marked with *). (B): Fatty acid ethyl esters result from the condensation of fatty acyl-CoA with ethanol, by action of the orthologs of genes EEB1 and EHT. Substituted ethyl esters may be produced by action of the orthologs of genes MGL2 and YJU3 (Marullo et al., 2021). In A and B *S. cerevisiae* genes in grey boxes; orthologs in *I. terricola* are indicated in italics next to the enzyme name. C) Esters produced by *I. terricola* during Viura must fermentation, quantified by HS-SPME/GCMS as duplicates by comparison with standards and expressed as the value of the area ratio (sample/standard). (D): Lactones produced by *I. terricola* during Viura must fermentation, quantified by HS-SPME/GCMS as duplicates by comparison with standards and expressed as the value of the area ratio (sample/standard).

exception, since the enzymes involved were identified in its genome: acyl-CoA oxidase (g4331.t1), enoyl-CoA hydratase (g1315.t1), hydroxy acyl-CoA dehydrogenase (g1315.t1), and ketoacyl-CoA thiolase (g2686.t1).

7. *I. terricola* β-glucosidases

Given our previous promising results in relation to the BGL activity of *I. terricola* 0621 strain on the glycosylated aroma precursors of musts and wines, we decided to continue the search for other β-glucosidases for their further characterisation and oenological application. Ten proteins were identified as putative β-glucosidases. The classification of these ten enzymes according to specific families was confirmed (Table 3): four of them belong to the GH17 family (g989.t1, g1025.t1, g2300.t1, g4410.t1), three to the GH132 (945, 3296, 3698), two to the GH5 (g1395.t1, g.4180.t1) and the last to the GH3 family (4598). The g4180 gene codes for the previously characterised β-glucosidase (González-Pombo *et al*., 2011).

DISCUSSION

Knowledge of non-conventional yeasts and their biotechnological use has increased considerably over the last 10 years. However, there is still a considerable lack of data on these yeasts compared to the conventional wine yeast *S. cerevisiae*. In this paper, we present the whole-genome sequencing of the 0621 strain of *I. terricola.* The assembly revealed a 12.5 Mb genome with a GC content of 36.4 %, which encodes 5377 proteins. Those data are in accordance with genomic data for other yeasts (Douglass *et al*., 2018; Giorello *et al*., 2018; Seixas *et al*., 2019). When assigned to Brite categories, the number of proteins in each category is comparable to those of related organisms, which supports the quality of the genome annotation. The analysis of the genome allowed us to determine the strain´s metabolic traits relevant for winemaking, as well as for its ability to grow on the different nutrients available in musts, adapt to stressful conditions, and generate substances that can confer desirable properties to the wine.

We were able to identify the catabolic pathways and the transporters for an ample range of carbon sources, which suggests that *I. terricola* adapts by utilising different available nutrients. It is worth mentioning that, as in other yeasts and fungi, we found homologs of the key regulators of carbon catabolite repression (Gancedo, 1998; Kayikci and Nielsen, 2015): g4645 is orthologous to *S. cerevisiae MIG1* and g1591.t1 to *SNF1*. The enzymes involved in the catabolism of hexoses (except galactose), pentoses, monocarboxylic acids, lipids and glycerol were identified in the genome of *I. terricola*. As already mentioned, most of the genes identified in *S. cerevisiae* as being part of the fermentation process are present in *I. terricola*, but some of those that were found to be essential or important for a successful and well-timed fermentation are absent in the latter. Our experimental data supports this observation, since, in contrast to *S. cerevisiae*, *I. terricola* was not able to perform a complete fermentation, consuming only 25 % of total fermentable sugars (Supplementary Figure 2). Alternatively, or additionally, the lack of homologs of many of the genes involved in stress tolerance could explain this metabolic trait.

The putative ability of *I. terricola* to metabolise pentoses contrasts with *S. cerevisiae*, which is unable to grow on ribose, xylose and L-arabinose (Ruchala and Sibirny, 2021). However, we were not able to identify the permeases responsible for the transport of these sugars, although it has been shown that they can enter the cell through transporters of the HXT hexose transporter family and through the

TABLE 3. β-glucosidases identified in the *I. terricola* genome.

Gene	Family*	Protein Domains**		BLAST Swiss-Prot***	
		Pfam	InterPro	Description	Organism
g4598.t1	GH3	Glycosyl hydrolases family 3 N terminal domain	Glycoside hydrolase superfamily	Beta-hexosaminidase	*Photobacterium profundum*
g1395.t1	GH5	Cellulase (glycosyl hydrolase family 5)	Glycosyl hydrolase family 5	Glucan 1,3-beta-glucosidase	*Saccharomyces cerevisiae*
g4180.t1		Cellulase (glycosyl hydrolase family 5)	Glycoside hydrolase, family 5, conserved site	Glucan 1,3-beta-glucosidase	*Saccharomyces cerevisiae*
g989.t1	GH17	Glycosyl hydrolases family 17	Glycosyl hydrolase family 17	Glucan 1,3-beta-glucosidase	*Saccharomyces cerevisiae*
g1025.t1		Glycosyl hydrolases family 17	Glycosyl hydrolase family 17	Probable family 17 glucosidase	*Saccharomyces cerevisiae*
g2300.t1		-	Glycoside hydrolase superfamily	Probable family 17 glucosidase	*Saccharomyces cerevisiae*
g4410.t1		-	Glycoside hydrolase superfamily	Probable family 17 glucosidase	*Pichia angusta*
g945.t1	GH132	Beta-glucosidase (SUN family)	SUN family	Uncharacterized protein	*Saccharomyces cerevisiae*
g3296.t1		Beta-glucosidase (SUN family)	SUN family	Probable secreted beta-glucosidase	*Saccharomyces cerevisiae*
g3698.t1		Beta-glucosidase (SUN family)	SUN family	Probable secreted beta-glucosidase	*Saccharomyces cerevisiae*

*Classification of BGLs based on the CAZy database. **Domains of *I. terricola* annotated proteins in the Pfam and InterPro databases. **Identification by BLAST search of *I. terricola* annotated proteins in the Swiss-Prot database. For details see Methods section.

galactose permease GAL2 (Nijland and Driessen, 2020). *I. terricola* has a gene that has homology with GAL2, despite the enzymes for the catabolism of galactose being absent. *I. terricola* also has five homologs of HXT genes, which is a low number compared to the 20 HXT genes present in *S. cerevisiae* (Perez *et al*., 2005). Thus, the potential ability of *I. terricola* to metabolise pentoses makes it possible to study its capacity to synthesise chemicals and biomass from pentoses, a topic that has also attracted much attention in recent years (Ruchala and Sibirny, 2021).

As already shown, *I. terricola* is able to grow on glycerol as the sole carbon source in aerobic conditions, and its genome harbours genes encoding the enzymes of the DHA and the GA glycerol catabolic pathways (Klein *et al*., 2017). The latter was suggested by Tom *et al*. (1978) as a way of carrying out glycerol dissimilation in the filamentous fungus *Neurospora crassa;* however, it has not yet been reported for any yeast species. It would be interesting to evaluate *in vitro* the activities of the enzymes involved in these pathways in extracts of *I. terricola* on different carbon sources (Tom *et al*., 1978). As previously mentioned, the functionality of the DHA pathway relies on a putative NAD^+-dependent activity of the g3745.1 gene, or on the existence of another enzyme with such an activity, which remains to be determined. Three genes predicted to code for glycerol transporters were identified.

Regarding the use of nitrogen sources, our findings suggest that *I. terricola* has adapted to transport and catabolise the variable and broad spectrum of assimilable nitrogenous compounds of which wine musts are composed. The key regulators of nitrogen catabolite repression (Magasanik and Kaiser, 2002) were identified in its genome: g5180.t1 is orthologous to URE2, g1046.t1 to GLN3 and g3408.t1 to GAT1 DAL80 and DEH1. However, those which are the preferred sources in pure cultures or in combined cultures with *S. cerevisiae* still need to be determined. Among the identified transporters, the high number of GABA transporters present in the genome of *I. terricola* is noteworthy. This compound may account for up to 20 % of the assimilable nitrogen in grape juices. Moreover, in *S. cerevisiae* the assimilation of GABA has favourable effects on yeast growth, fermentation rate and glycerol production under scarce nitrogen conditions, and is a source of succinate, which may contribute to pH reduction (Bach *et al*., 2009).

During wine production, the participating microorganisms must generate appropriate responses to cope with the variety of environmental stresses to which they are exposed, like elevated sugar and ethanol concentration, or different temperatures from those which are optimal for growth. According to our results, only 70-75 % of the *S. cerevisiae* resistance genes involved in high sugar and high ethanol concentrations, have homologs with *I. terricola*. What is more surprising is that even genes identified as relevant or essential determinants for survival are missing. In the case of high ethanol resistance, no homologs of *CWC25* and *SPP381* could be found. These two genes are linked to the splicing of mRNAs, which in *S. cerevisiae* is thought to be a way of eliciting rapid and specific changes in gene expression in response to environmental stress (Bergkessel *et al*., 2011). Another relevant resistance gene missing in *I. terricola* is *FPS1*, which encodes an aqua glycerol protein involved in the control of intracellular levels of glycerol, as well as the maintenance of ethanol intracellular levels during fermentation (Teixeira *et al*., 2009; Yoshikawa *et al*., 2009). Orthologs of *MSN2/MSN4*, which are crucially regulated in response to high-glucose tolerance (Brion *et al*., 2016; Gasch, 2007), are missing in *I. terricola*. MSN2 and MSN4 bind to stress response elements (STRE) present in the promoters of a number of the genes involved in the response to environmental stress (Martínez-Pastor *et al*., 1996). MSN2 and/or MSN4 are also missing or little conserved in other NS-yeasts, like members of the *Hanseniaspora* genus, or *Lachancea kluyveri* (Brion *et al*., 2016; Seixas *et al*., 2019). Nevertheless, we were able to identify the orthologs of *HOG1* and *PBS2* (g3226.t1 and g4853.t1 respectively), which lay upstream in the HOG hyperosmotic stress signalling pathway and are among the *S. cerevisiae* genes involved in high-glucose resistance. Taken together, these results reinforce the idea that there are important differences in the way yeast species respond to environmental stresses during winemaking (Brion *et al*., 2016; Seixas *et al*., 2019). However, as previously stated, *I. terricola* stops growing before fermentation is complete. It cannot be ruled out that the lack of the aforementioned genes renders this yeast more susceptible to environmental stress.

The amount and length of lipids have a strong impact on membrane thickness, dynamics and fluidity characteristics, which is relevant in stressful conditions (Henderson *et al*., 2013). In the case of *I. terricola,* we found all the central pathways of membrane and reserve lipid synthesis, such as the synthesis of phosphatidic acid, the precursor in the synthesis of membrane phospholipids and neutral lipids. Under certain nutritional conditions, these lipids are stored in cytoplasmic lipid droplets, which indicates that there is a crossroads between membrane biogenesis and lipid storage (Ejsing *et al*., 2009). In fact, lipid droplets which store neutral lipids, such as triglycerides and steryl esters, are important in the cell as they supply of energy and membrane lipids and also regulate cell longevity (Goldberg *et al*., 2009; Welte and Gould, 2017).

Our experimental results show that *I. terricola* produces several active flavour compounds, like aliphatic and aromatic alcohols, acetate and ethyl esters and γ-nonalactone.We were able to identify the genes and pathways involved in the synthesis of these compounds. In some cases, orthologs of the *S. cerevisiae* genes involved in the pathways were identified; in others, the involvement of genes different to those which catalyse a certain step in the budding yeast, but that have the same activity, is likely. For example, it is possible that the alcohol acetyl-transferase activity encoded by *S. cerevisiae ATF1* and *ATF2* genes, which are involved in the production of acetate esters, can be fulfilled by the products of g20.t1 and g3217.t1, which possess an alcohol acetyltransferase/ N-acetyltransferase domain. However, these genes are

phylogenetically closer to *SLI1*, a gene shown to be involved in resistance to the sphingolipid biosynthesis inhibitor drug myriocin in the budding yeasts (Momoi *et al*., 2004). Hence, further research must be conducted in order to confirm these possible pathways.

I. terricola 0621 was selected to add aromatic complexity to Muscat and Tannat wines because of its outstanding BGL activity, which increases the level of free monoterpenes and norisoprenoids (de Ovalle *et al*., 2018; González-Pombo *et al*., 2011). However, BGLs are inactivated in acidic pH and fermentation conditions, which limits the use of the *I. terricola* 0621 strain in mixed cultures to increase the varietal aroma during must fermentation. As an alternative, it is possible to consider the heterologous production of these BGLs. Indeed, our previous findings related to *I. terricola* g4180.t1 and the remaining BGL genes identified in the present study motivated the characterisation of the oenological properties of the encoded enzymes. This will eventually allow us to design new application strategies for aroma production in vinification, such as the cloning and expression of *S. cerevisiae*.

To sum up, this study constitutes an example of how the availability and exploration of the genomes of non-conventional native strains can provide tools for the study of metabolic mechanisms not yet fully understood, and of the reservoir of genes that could eventually be used to generate recombinant strains or for the biotechnological production of the enzymes.

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