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# Physiological and biochemical characteristics of the ethyl tiglate production pathway in the yeast *Saprochaete suaveolens*

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## Abstract

A yeast identified as *Saprochaete suaveolens* was investigated for its capacity to produce a large panel of flavouring molecules. With a production of 32 compounds including 28 esters, *S. suaveolens* seems to be a good producer of fruity flavours and fragrances and especially of unsaturated esters, such as ethyl tiglate. Physiological and biochemical analyses were performed in this study in an attempt to comprehend the metabolic route to the formation of this compound. We show that the accumulation of ethyl tiglate by *S. suaveolens* is specifically induced by isoleucine. However, and contrary to *S. cerevisiae*, which harbours a classical Ehrlich pathway leading to the production of 2-methylbutanol from isoleucine, our results provide phenotypic and enzymological evidence of ethyl tiglate biosynthesis in *S. suaveolens* through the catabolism of this amino acid by the  $\beta$ -oxidation pathway, which generates tiglyl-CoA as a probable intermediate. A kinetic analysis of this flavour molecule during growth of *S. suaveolens* on glucose and isoleucine showed a phase of production of ethyl tiglate that culminated concurrently with isoleucine exhaustion, followed by a disappearance of this compound, likely due to reassimilation by the yeast.

**Keywords:** Ehrlich pathway; flavours and fragrances; esters;  $\beta$ -oxidation; *Saprochaete suaveolens*; ascomycetes

## Introduction

With renewed interest for natural products during the last decade, there is an increasing need to produce natural molecules using microbial systems for many applications. Flavours and fragrances are important compounds in the food, cosmetic, pharmaceutical and chemical industries (Pinotti *et al.*, 2006; Schrader, 2007). In 2013, the worldwide market of the flavour industry was estimated as 24 billion US\$ (<http://www.leffingwell.com>,

accessed in June 2014). Disadvantages of the conventional processes of production, such as extraction of plant material and chemical synthesis, has recently motivated both academic laboratories and several industrial companies to identify microbial sources that may naturally produce these molecules and develop biotechnological processes to optimize the production and productivity of them, or to engineer microbial systems to produce new added-value chemicals (Keasling, 2012).

The microbial production of flavouring molecules has been extensively studied during recent decades and many reviews in this field have been published (Krings and Berger, 1998; Abbas, 2006; Berger, 2009; Buzzini and Vaughan-Martini, 2006; Feron *et al.*, 1996; Schrader, 2007; Mdaini *et al.*, 2006; Dastager, 2009; Pires *et al.*, 2014; Styger *et al.*, 2011; Löser *et al.*, 2014; Kim *et al.*, 2014; Cheon *et al.*, 2014). Biotechnological production of flavour compounds is nowadays considered to be a mature discipline in the industry, with an estimated 100 molecules in the market produced by enzymatic or microbial processes (Schrader, 2007). Among them, molecules such as vanillin,  $\gamma$ -decalactone, 2-phenylethanol and raspberry ketone are considered to be high added-value products (Serra *et al.*, 2005; Schrader, 2007; Hua and Xu, 2011; Priefert *et al.*, 2001).

Ethyl tiglate, also known as ethyl (*E*)-2-methylbut-2-enoate, is a branched-chain alkyl ester naturally found in some organisms, such as *Mangifera indica* L. (mango) (Pino *et al.*, 2005), *Malus domestica* Borkh. (red apple) (Matich and Rowan, 2007), *Cydonia vulgaris* (quince) (Schreyen *et al.*, 1979), *Pyrus serotina* (Asian pear) (Takeoka *et al.*, 1992) and *Pelargonium graveolens* (geranium rosat) (<http://www.perfumerflavorist.com>, accessed in May 2014). This FEMA-GRAS (Flavor and Extract Manufacturers Association; generally recognised as safe) compound is characterized by a fruity odour and is mainly used in perfumery to add unusual top notes, particularly for the non-floral fragrance types (Arctander, 1969). This compound is also used in the food industry for the production of alcoholic beverages, such as rum, and for tobacco flavouring (<http://www.perfumerflavorist.com>, accessed February 2014). Ethyl tiglate is also described by entomologists as an aggregation pheromone in some *Drosophila* species (Bartelt *et al.*, 1985, 1988; Moats *et al.*, 1987); mature males of *D. borealis* and *D. littoralis* produce these pheromones that attract both males and females. Data regarding the production of ethyl tiglate by microorganisms is rather scarce. Pinotti *et al.* (2006) reported the production of ethyl tiglate by the yeast *Geotrichum candidum* under some specific growth conditions. An extensive analysis carried out by another group did not identify ethyl tiglate among the volatile compounds produced by *Saprochaete suaveolens* when cultivated on cassava waste water (Takeoka *et al.*, 1992; Damasceno *et al.*, 2003).

Finally, two European patents refer to the production of ethyl tiglate by some microbial strains, including *Geotrichum klebahnii* (*Geotrichum penicillatum*, CBS 62774) and *Saprochaete suaveolens* (*Geotrichum suaveolens*, CBS 38236 and *Geotrichum fragrans* ATTC 11247) (Farbood *et al.*, 1987, 1992).

The metabolism of ethyl tiglate has never been described in yeast and has only been described in red apple (Matich and Rowan, 2007); the results of the latter study suggested that the synthesis of ethyl tiglate in fruit occurred from isoleucine metabolism via a modified Ehrlich pathway. This pathway is well known for the production of higher alcohols and related flavour compounds, such as 2-methylbutanol, 2-methylbutanoic acid, ethyl 2-methylbutanoate, 2-methylbutyl ethanoate or 2-phenylethanol (Hazelwood *et al.*, 2008).

In the present study we focused on the production of ethyl tiglate by the yeast *Saprochaete suaveolens*, a yeast also known as *Geotrichum fragrans*, which belongs to the *Saccharomycetes* class of microorganisms (De Hoog and Smith, 2004, 2011) and which we isolated from Pitaya fruit (*Hylocereus polyrhizus*) in Reunion Island as part of a screening programme of yeasts capable of producing volatile aromatic molecules of particular interest for industry. We show here that the main precursor of ethyl tiglate is isoleucine, and provide evidence that the metabolic pathway leading to this compound is distinct from the Ehrlich pathway, which converts this amino acid into 2-methylbutanol (amyl alcohol) in other yeasts, such as *Saccharomyces cerevisiae*.

## Materials and methods

### Yeast strains

The strain GEC0 of *Saprochaete suaveolens* (*Geotrichum fragrans*) (De Hoog and Smith, 2004, 2011) used in this study was isolated from Pitaya fruits (*Hylocereus polyrhizus*) in the area of Saint Paul in Reunion Island (France). The microorganism was first identified using an API 20C AUX strips (bioMérieux) and further confirmed by 26S rDNA D1/D2 sequencing, identified according to Kurtzman and Robnett (1997). This study was performed by the Centre International de Ressources Microbiennes (CIRM, INRA, Paris

Grignon). The D1-D2 sequence of the isolated strain showed a difference of one nucleotide from the reference strain of *S. suaveolens* CBS 152.25. Wild-type *Saccharomyces cerevisiae* CEN.PK 122-2N (van Dijken *et al.*, 2000) yeast strain was used as reference strain in this work.

### Culture media and growth kinetics

Cells were stored in cryogenic vials at  $-80\text{ }^{\circ}\text{C}$  and refreshed on YPD agar slants containing 20 g/l glucose ( $\alpha$ -D-glucose, anhydrous, Sigma-Aldrich), 20 g/l peptone (Becton-Dickinson), 10 g/l yeast extract (Biokar Diagnostics) and 15 g/l agar (agar-agar for microbiology; Merck). The cells were refreshed at  $28\text{ }^{\circ}\text{C}$  for 48 h prior to their utilization.

For qualitative study of flavour compounds produced by the yeast strains, a loop of fresh cells was spread on inclined YPD slants incubated at  $28\text{ }^{\circ}\text{C}$  for 48 h. The tubes were sealed after 24 h of incubation.

For quantitative experiments and enzymatic assays, experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml YNB–glucose medium (0.67% w/v Yeast Nitrogen Base without Amino Acids, 2% w/v glucose, Sigma-Aldrich). Liquid cultures were inoculated at an initial optical density measured at 600 nm of 0.1 U with cells precultivated in YNB–glucose and incubated at  $28\text{ }^{\circ}\text{C}$  and 110 rpm for 24 h. Growth cultures were incubated under the same conditions for 120 h. When appropriate, 1 g/l L-isoleucine (Sigma-Aldrich) was added to the medium, as indicated in the legends to figures. Cell growth was measured at regular intervals, using a Jenway Spectrophotometer 6450 set to 600 nm ( $\text{OD}_{600}$  = optical density at 600 nm). Quantitative and qualitative experiments were performed in independent triplicates.

### Qualitative analysis of volatile metabolites

Isolation and characterization of volatile metabolites was performed using solid-phase micro-extraction (SPME), followed by gas chromatography–mass spectrometry (GC–MS) analysis. Prior to analysis, 25  $\mu\text{l}$  octanol (1 g/l in ethanol) was added into the sealed vials containing the yeast culture, as an internal standard. The headspace of the inclined cultures on YPD was subjected to SPME analysis, using a 2 cm-long fibre coated with 50/30  $\mu\text{m}$  divinylbenzene/Carboxen<sup>TM</sup> on polydimethylsiloxane,

bonded to a flexible fused silica core (Supelco) (Buzzini *et al.*, 2005). The fibre was exposed to the headspace for 15 min at  $30\text{ }^{\circ}\text{C}$  and inserted into the injection port of the GC–MS (Agilent Technologies 6890N Network GC system) for thermal desorption at  $270\text{ }^{\circ}\text{C}$  for 15 min. Metabolites were separated by gas chromatography (GC) on a SPB5 column ((Supelco, 60 m x ...) 60 m  $\times$  0.32 mm, 0.25  $\mu\text{m}$  film thickness), coupled to a mass spectrometer (Agilent Technologies 5973 Network mass-selective detector). The carrier gas (He) was set at a flow rate of 0.8 ml/min. The column temperature was maintained at  $45\text{ }^{\circ}\text{C}$  for 2 min, raised to  $230\text{ }^{\circ}\text{C}$  at  $4\text{ }^{\circ}\text{C}/\text{min}$  and finally kept at this temperature for 12 min. Volatile compounds were identified by comparing their mass spectra with the NIST and Wiley mass spectral databases and their Kovàts retention indexes with those from the NIST retention index library (<http://webbook.nist.gov/chemistry>, accessed May 2014).

### Quantitative assay of ethyl tiglate

The metabolites in the culture medium were extracted by liquid–liquid extraction. Samples containing 475  $\mu\text{l}$  fermentative medium were supplemented with 25  $\mu\text{l}$  octanol (1 g/l in ethanol). The mixture was then extracted using 500  $\mu\text{l}$  dichloromethane and the aqueous phase separated by centrifugation at  $14\ 000\times g$  for 5 min. Quantitative determination of ethyl tiglate was carried out by GC (Varian 430), using a CP-WAX 52 CB (25 m  $\times$  0.25 mm, 0.2  $\mu\text{m}$  film thickness) column, coupled to a flame ionization detector (FID). Injector and detector were set at  $230\text{ }^{\circ}\text{C}$  and  $250\text{ }^{\circ}\text{C}$ , respectively; 0.2  $\mu\text{l}$  organic phase of each sample was injected for analysis. The carrier gas (He) was set at a flow rate of 0.8 ml/min. The column temperature was maintained at  $45\text{ }^{\circ}\text{C}$  for 2 min, raised to  $230\text{ }^{\circ}\text{C}$  at  $4\text{ }^{\circ}\text{C}/\text{min}$  and finally kept at this temperature for 12 min. Ethyl tiglate and 2-methylbutanol used for the response factor curve and as a standard were purchased from Sigma-Aldrich.

### HPLC analysis of sugars and amino acids

The concentration of glucose and isoleucine was measured using an Ultimate 3000 HPLC system (Dionex) coupled to a Varian 380 LC evaporative light-scattering detector (Raessler, 2011; Noga *et al.*, 2013) (evaporation temperature  $50\text{ }^{\circ}\text{C}$ ,

nebulization temperature 50 °C, N<sub>2</sub> flow 1.6 slm Standard liter per minute). For glucose analysis, separation was performed using a Hi-Plex Ca column (Agilent, 7.7×300mm, 8µm film thickness) under isothermal conditions (85 °C). The injection volume of filtered samples (through 0.45 µm Sartorius filters) was 10 µl and MilliQ water was used as mobile phase, at a flow rate of 0.6 ml/min. Isoleucine was analysed using an Atlantis Silica HILIC column (100 Å, 3 µm, 2.1 mm×100 mm) under isothermal conditions (30 °C). 2 µl filtered samples (through 0.20 µm Sartorius filters) were injected into the chromatographic system and methanol:water (80:20 v/v) was used as the mobile phase, at a flow rate of 0.8 ml/min.

### Preparation of crude extracts and assays of enzymatic activities

Cells were collected by centrifugation at 1000×g at 4 °C, washed twice in demineralized water and stored at −20 °C until use. Cell-free extracts from early stationary phase culture samples were prepared using a Qiagen Retsch TissueLyser II (3 min, 30 Hz). The buffer was 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA, 100 mM KCl and 1 mM DTT. The extract was centrifuged at 4 °C for 5 min at 1000×g in an Eppendorf tabletop centrifuge and the supernatant was used for determination of enzyme activities and protein concentration. Pyruvate and 2-oxo-acid decarboxylase (DC) activity on  $\alpha$ -keto acids, pyruvate and 2-oxo-3 methylpentanoic acid was determined according to Ullrich (1970), with some modifications. The medium contained 50 mM citrate buffer, pH 6.2, 100 mM KCl, 0.2 mM DTT, 0.5 mM TPP, 5 mM MgSO<sub>4</sub>, 0.1 mM NADH and 5 U/ml yeast alcohol dehydrogenase (Sigma-Aldrich). The reaction was started by the addition of 50 mM pyruvate or 0.2 mM 2-oxo-3-methylpentanoic acid (determined at saturated concentrations of substrate), and oxidation of NADH was followed spectrophotometrically at 340 nm. Alcohol dehydrogenase (ADH) was assayed in a mixture containing 50 mM citrate buffer, pH 6.2, 100 mM KCl, 0.2 mM DTT, 0.5 mM TPP, 5 mM MgSO<sub>4</sub> and 0.1 mM NADH, according to Hansen and Hensgens (1994). The reaction was measured spectrophotometrically following the oxidation of NADH at 340 nm after addition of 2 mM 2-methylbutanal. ADH was also assayed in a mixture containing 20 mM HEPES buffer, pH 7.6,

2 mM DTT and 1.5 mM NAD; the reaction was started by the addition of 40 mM ethanol. The reduction of NAD to NADH was followed at 340 nm. Aldehyde dehydrogenase (ALDH) was assayed in a mixture containing 50 mM citrate buffer, pH 6.2, 100 mM KCl, 0.2 mM DTT, 0.5 mM TPP, 5 mM MgSO<sub>4</sub> and 1.5 mM NAD, as described by Tamaki *et al.* (1982); the reaction was started by the addition of 2 mM 2-methylbutanal. The reduction of NAD to NADH was followed at 340 nm. The branched-chain  $\alpha$ -keto acid dehydrogenase (BCKAD) assay was performed following the reduction of NAD to NADH at 340 nm (Patston *et al.*, 1988) in 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA, 0.2 mM DTT, 0.5 mM TPP, 5 mM MgSO<sub>4</sub>, 1.5 mM NAD and 0.5 mM CoA-SH; the reaction was started by the addition of 0.2 mM 2-oxo-3-methylpentanoic acid. The acyl-CoA dehydrogenase (ACyD) activity was determined spectrophotometrically following the reduction of DCIP (2,6-dichlorophenolindophenol) into DCIPH<sub>2</sub> at 655 nm (Ikeda and Tanaka, 1988). The reaction was performed in 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA, 100 mM KCl, 0.1 mM FAD and 0.5 mM DCIP; the reaction was started by the addition of 0.2 mM 3-methylbutanoyl-CoA. For the assay of alcohol acyltransferase activity, *p*-nitrophenol production (Schermers *et al.*, 1976) was followed at 415 nm in a medium containing 50 mM potassium phosphate buffer, pH 7.4, 2 mM EDTA and 100 mM KCl; the reaction was started by the addition of 2 mM *p*-nitrophenylbutyrate. A blank without addition of the corresponding substrate was run in parallel for each sample in all the enzymatic assays. Protein concentration was determined at 550 nm by a modified Bradford (1976) method, with bovine serum albumin (BSA; Sigma-Aldrich) as the standard. All assays were performed on three biological replicates.

## Results and discussion

### Volatile organic compounds production by *S. suaveolens* and *S. cerevisiae* on YPD

The volatile organic compounds produced by the yeast *S. suaveolens* (GECO) and *S. cerevisiae* strain CENPK122-2N after 48 h of growth on YPD medium were determined by headspace GC-MS (Table 1). This chemical analysis allowed

**Table 1.** Volatile organic compounds (VOCs) produced by the yeast *Saprochaete suaveolens* and *Saccharomyces cerevisiae* after 48 h of growth on YPD agar medium and identified by headspace GC–MS

Volatile compounds	Odour type <sup>a</sup>	Odour threshold <sup>a,b</sup> (µg/l)	RRI exp <sup>c</sup>	RRI th <sup>d</sup>	VOCs production (µg/l)	
					<i>S. suaveolens</i>	<i>S. cerevisiae</i>
<i>Alcohols</i>						
1-Butanol	Malted, solvent	500	733	654	10 ± 7	95 ± 48
2-Methylpropanol	Alcohol, malted	7000	661	607	nd	115 ± 49
2-Methylbutanol	Malted, solvent	1200	1105	727	18 ± 26	194 ± 100
3-Methylbutanol	Malted, alcohol, fruity	250–300	736	725	92 ± 17	355 ± 198
2-Phenylethanol	Flowers, honey, rose	750–1100	1065	1104	152 ± 57	175 ± 90
<i>Esters</i>						
Ethyl acetate	Fruity	5–5000	620	600	233 ± 109	58 ± 25
Ethyl propanoate	Banana, apple	10	709	694	18 ± 12	8 ± 5
Ethyl butanoate	Fruity, pear, pineapple	1	797	791	106 ± 71	46 ± 40
Ethyl 2-methylpropanoate	Sweet, fruity, banana, apple	0.1	755	751	79 ± 51	8 ± 8
Ethyl but-2-enoate			841	833	51 ± 4	nd
Ethyl 2-methylbutanoate	Fruity, green, apple, floral	0.1–0.3	847	842	270 ± 137	17 ± 11
Ethyl tiglate	Fruity	65	935	939	575 ± 62	nd
Ethyl 3-methylbutanoate	Fruity, blueberry	0.023	850	856	1064 ± 545	35 ± 39
Ethyl 3-methylbut-2-enoate		17–38	919		17 ± 1	nd
Butyl acetate	Fruity, pear, pineapple	66	812	810	nd	17 ± 15
Butyl tiglate		2.9–6.7	1125		5 ± 1	nd
Butyl 2-methylbutanoate			1035	1033	2 ± 1	nd
Butyl 3-methylbutanoate			1039	1035	23 ± 7	5 ± 2
Pentyl propanoate			964		nd	7 ± 5
Pentyl 3-methylbutanoate	Fruity		1098	1108	62 ± 27	nd
2-Methylpropyl acetate		66	771	763	17 ± 13	nd
2-Methylpropyl butanoate			951	946	12 ± 5	nd
2-Methylpropyl tiglate			1084		112 ± 30	nd
2-Methylpropyl 2-methylpropanoate	Fruity	30	909	902	29 ± 7	nd
2-Methylpropyl 2-methylbutanoate			995	995	61 ± 18	nd
2-Methylpropyl 3-methylbutanoate			997		295 ± 140	4 ± 5
2-Methylbutyl acetate		1	875	869	9 ± 8	nd
2-Methylbutyl butanoate			1052	1047	2 ± 0	3 ± 1
2-Methylbutyl 2-methylpropanoate			1009	1002	11 ± 3	nd
3-Methylbutyl acetate	Pear, banana, sweet, fruity	2	873	867	22 ± 17	135 ± 106
3-Methylbutyl propanoate	Apricot, pineapple		964		2 ± 1	nd
3-Methylbutyl butanoate	Apricot, pineapple		1049	1044	5 ± 3	11 ± 3
3-Methylbutyl 2-methylbutanoate			1091	1101	47 ± 20	2 ± 1
3-Methylbutyl tiglate			1185		100 ± 40	nd
3-Methylbutyl 3-methylbutanoate			1096	1094	258 ± 138	4 ± 2
2-Phenylethyl acetate			1249	1225	nd	7 ± 4

<sup>a</sup>According to Leffingwell and Leffingwell, 1991; Molimard and Spinnler, 1996; Takeoka et al., 1998; Kirchoff and Schieberle, 2002; Christoph and Bauer-Christoph, 2007; Chen et al., 2006; Czerny et al., 2008.

<sup>b</sup>Odour threshold expressed as µg/l water.

<sup>c</sup>Calculated relative retention index on non-polar column.

<sup>d</sup>Relative retention index on non-polar column (<http://webbook.nist.gov/chemistry/>).

Data are presented in µg/l in the headspace and as an average of three independent biological assays (± SD). nd, not detected.

the identification of 32 molecules produced by *S. suaveolens* and 21 molecules by *S. cerevisiae*. These molecules could be classified into two main groups, esters and alcohols. *S. suaveolens* mostly produced esters (28 of 32 molecules), with 15 found only in cultures of this yeast species and not of *S.*

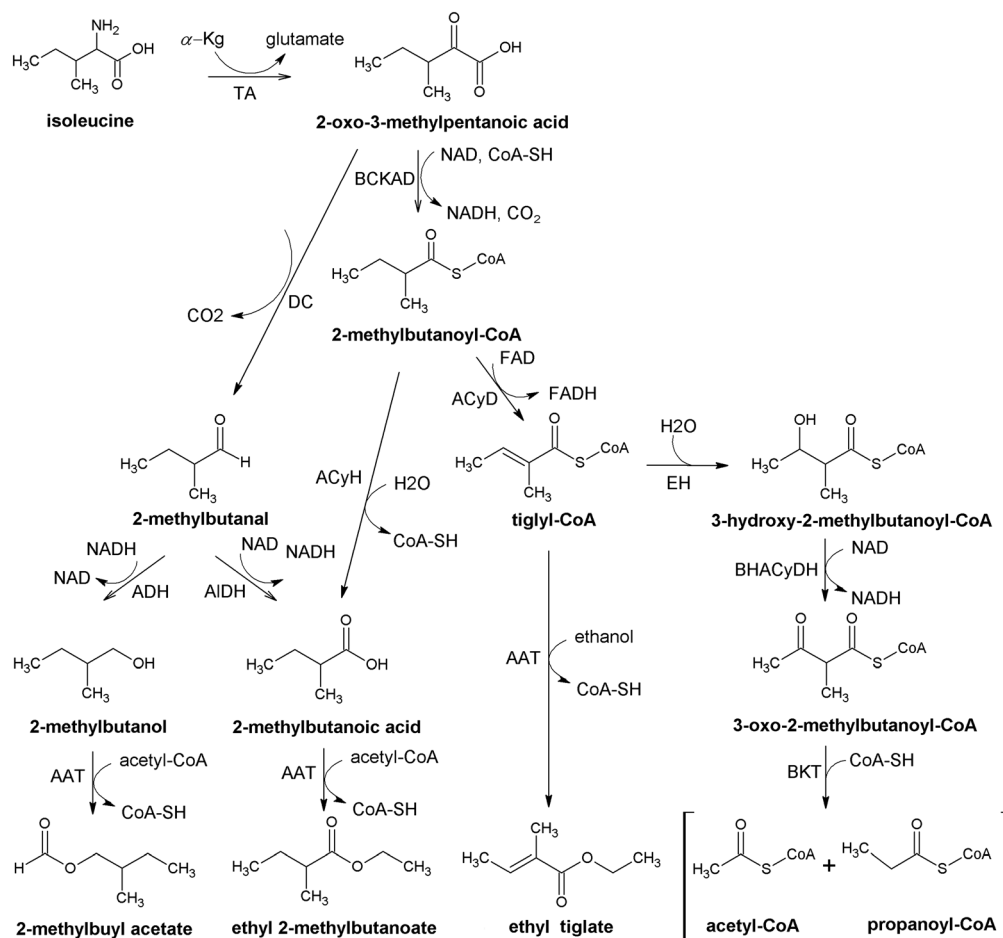
*cerevisiae*. The most abundant esters produced by *S. suaveolens* were ethyl 3-methylbutanoate (ethyl isovalerate), ethyl (*E*)-2-methylbut-2-enoate (ethyl tiglate), 2-methylpropyl 3-methylbutanoate (2-methylpropyl isovalerate) and 3-methylbutyl 3-methylbutanoate (isoamyl isovalerate), whose

values were in the range 258–1065 µg/l in the head-space of cultures. These esters were also detected in *G. candidum* (Mdaini *et al.*, 2006) and were classified by Verstrepen *et al.* (2003) as having strong fruity notes with low odour thresholds. In addition, some of these esters, such as ethyl tiglate, could have potential applications as flavouring agents (Christoph and Bauer-Christoph, 2007). On the other hand, alcohols (1-butanol, 2-methylpropanol, 2-methylbutanol, 3-methylbutanol and 2-phenylethanol) were more abundantly produced by the yeast *S. cerevisiae*. Both yeast species also produced ethanol, but it was not quantified here because it co-eluted with the dilution solvent of the internal standard (data not shown). Altogether, these

results indicate that *S. suaveolens* has a greater ability to produce ester-type flavour compounds, and hence suggested more efficient alcohol acyltransferase activities in this yeast than in *S. cerevisiae*.

### Influence of metabolic precursors and kinetic production of ethyl tiglate in *S. suaveolens*

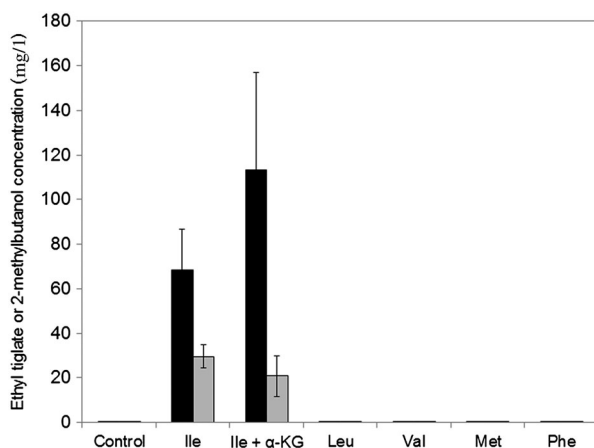
The metabolic pathway by which ethyl tiglate is produced by microbial cells has not yet been elucidated. However, as depicted in Figure 1, Matich and Rowan (2007) proposed that this volatile compound is produced from isoleucine through mitochondrial  $\beta$ -oxidation. According to this hypothesis, we investigated the effects on the production of ethyl tiglate



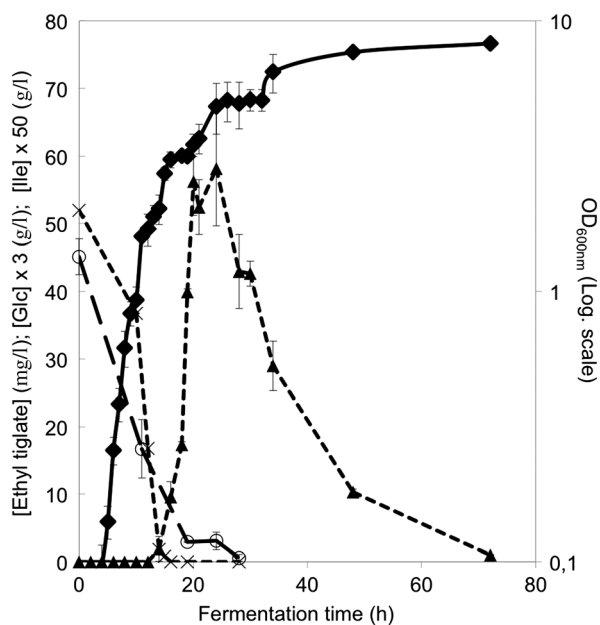
**Figure 1.** Proposed metabolic routes for the metabolism of isoleucine to some esters in the yeast *Saprochaete suaveolens* (adapted from Matich and Rowan, 2007): TA, transaminase; DC, decarboxylase; BCKAD, branched-chain  $\alpha$ -keto acid dehydrogenase; ACyH, acyl-CoA hydrolase; AAT, alcohol acyltransferase; ACyD, acyl-CoA dehydrogenase; AIDH, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; EH, enoyl Co-A hydratase; BHACyDH,  $\beta$ -acyl-CoA dehydrogenase; BKT,  $\beta$ -keto Co-A thiolase

of various amino acids added at 1 g/l to the culture medium. In agreement with this hypothesis, it is shown in Figure 2 that ethyl tiglate increased from <1 mg/l in the control experiment to >60 mg/l in YNB–glucose supplemented with 1 g/l isoleucine, whereas there was no production of ethyl tiglate upon addition of alanine, valine, leucine, methionine or phenylalanine. When  $\alpha$ -ketoglutarate was added together with isoleucine in the YNB–glucose medium, the concentration of ethyl tiglate increased further to 113 mg/l. A production of 2-methylbutanol at about half that of ethyl tiglate was also observed upon addition of isoleucine to the medium. These results, taken as a whole, clearly indicate that the Ehrlich pathway in *S. suaveolens* is fully functional and that ethyl tiglate production may likely occur through the  $\beta$ -oxidation of isoleucine, as has been suggested for the production of this flavour compound in apple (Matich and Rowan, 2007).

The kinetics of production of ethyl tiglate during fermentation in YNB glucose medium supplemented with 1 g/l isoleucine is reported in Figure 3. It can be seen that accumulation of ethyl tiglate began at the end of growth on glucose and reached its maximum at 24 h, concomitantly with the complete exhaustion of isoleucine. This result



**Figure 2.** Ethyl tiglate (black bars) and 2-methylbutanol (grey bars) production by *Saprochaete suaveolens*, as influenced by supplementation of the medium with isoleucine (Ile) alone or associated with the amino acceptor  $\alpha$ -ketoglutarate ( $\alpha$ -KG). Samples were harvested during stationary phase after glucose and isoleucine depletion. The cultures were performed in 50 ml of YNB–glucose (20 g/l) supplemented with Ile or Leu or Val or Met or Phe or Ile +  $\alpha$ -KG (1 g/l each),  $\text{pH}_{\text{initial}} = 5.5$  at  $T = 27^\circ\text{C}$  under agitation at 110 rpm. Tween 80 (surfactant, 1 g/l) was added to the medium in order to stabilize ethyl tiglate concentration in the medium



**Figure 3.** Ethyl tiglate production by *Saprochaete suaveolens* during growth on YNB–glucose medium; LOG biomass (—◆—), glucose (Glc; -- X --), isoleucine (Ile; —○—) and ethyl tiglate (—▲—) concentrations in the medium. The cultures were performed in 50 ml YNB–glucose (20 g/l) supplemented with Ile (1 g/l),  $\text{pH}_{\text{initial}} = 5.5$  at  $T = 27^\circ\text{C}$  under agitation at 110 rpm. For convenience, the concentrations of glucose and isoleucine were multiplied by 3 and 50, respectively

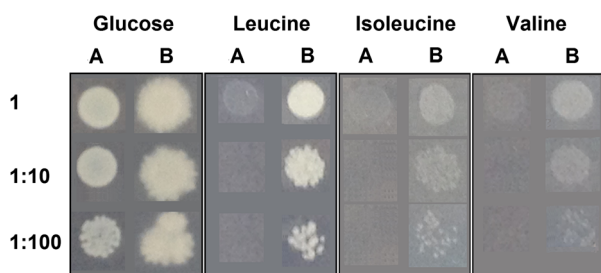
confirmed that the production of ethyl tiglate was fully dependent on the presence of isoleucine in the growth medium. Under these conditions, a productivity of 6.5 nmol/min/mg protein was estimated. This result indicated that the production of ethyl tiglate, as well as that of 10 mg/l 2-methylbutanol, arose as a metabolic consequence of the degradation of excess isoleucine. However, upon complete exhaustion of glucose and isoleucine, ethyl tiglate disappeared, which suggested the action of an esterase that converted ethyl tiglate into tiglic acid (or 2-methylbut-2-enoic acid). This latter compound could then be assimilated by the cells as a carbon source through the  $\beta$ -oxidation pathway (Figure 1).

### Phenotypic and enzymatic evidence that ethyl tiglate production required $\beta$ -oxidation in *S. suaveolens*

To provide evidence that the production of ethyl-tiglate in *S. suaveolens* implicates the  $\beta$ -oxidation pathway, we carried out two complementary



experiments. The first experiment was to show that this yeast species can grow on branched-chain amino acids, such as leucine, isoleucine or valine, because the  $\beta$ -oxidation of these amino acids leads to acetyl-CoA (plus propionyl-CoA in the case of isoleucine), which are then incorporated into the TCA cycle. As depicted in Figure 1, this pathway requires an oxidative decarboxylation of the keto-acids by a branched-chain  $\alpha$ -keto-acid dehydrogenase into the corresponding acyl-CoA. In the yeast *S. cerevisiae*, this  $\beta$ -oxidation of branched-chain amino acids does not take place (Dickinson *et al.*, 1997, 1998), which explains why this yeast species cannot grow on these branched-chain amino acids but converts them into fusel alcohols through the Ehrlich pathway (Hazelwood *et al.*, 2008). In agreement with this hypothesis, we show in



**Figure 4.** Growth of *Saccharomyces cerevisiae* (A) and *Saprochaete suaveolens* (B) strains on branched-chain amino acids as sole carbon source (YNB medium without amino acids). The cells were pregrown on YNB medium supplemented with 2% glucose and resuspended in MilliQ water at  $OD_{600}=3$ ; 5  $\mu$ l of each cell suspension and two serial 1:10 dilutions were spotted on YNB plates supplemented with 2 g/l glucose or 1 g/l leucine, isoleucine or valine as sole carbon source. The plates were incubated at 28 °C for 2 days

Figure 4 that *S. suaveolens* can grow very well on leucine but less efficiently on valine and isoleucine as sole carbon source present in the synthetic minimal medium, whereas *S. cerevisiae* was totally unable to grow on these media. The reason why growth of *S. suaveolens* on leucine was more efficient than on isoleucine and valine could be due to differences in  $\beta$ -oxidation metabolism. Indeed, while the first three reaction steps employed the same enzymes for the three amino acids (see Figure 1), the fourth step is catalysed by an enoyl-CoA hydratase in valine and isoleucine metabolism, whereas it is a 3-methylcrotonyl carboxylase that is required in the  $\beta$ -oxidation of leucine. Thus, one can hypothesize that enoyl-CoA hydratase is poorly active as compared to the carboxylase. This hypothesis would further explain the production of ethyl tiglate, which results from accumulation of tiglyl-CoA.

The second experimental evidence was to determine the activity of key enzymes in the Ehrlich and  $\beta$ -oxidation pathways and compared these activities between the two yeast species (Table 2). Under the conditions tested, the decarboxylase activity (DC) was 20 times more active in *S. cerevisiae* (2760 nM/min/mg protein) than in *S. suaveolens* (137 nM/min/mg protein) using pyruvate as substrate, and three-fold more active with 2-oxo-3-methylpentanoic acid. In addition, alcohol dehydrogenase acting on ethanol was much more active in *S. cerevisiae* than in *S. suaveolens*, but similar activity was found in both yeasts using 2-methylbutanal as substrate. Finally, the absence of activity of aldehyde dehydrogenase in both strains indicated a preference to use the reductive

**Table 2.** Activities of some enzymes involved in the Ehrlich and  $\beta$ -oxidation pathways in *Saprochaete suaveolens* and *Saccharomyces cerevisiae*

	Enzyme activities (nM/min/mg protein)							
	DC		ADH					
	On pyruvate	On 2-oxo-3-methylpentanoic acid	On ethanol	On 2-methylbutanal	AIDH	BCKAD	ACyD	AAT
<i>S. suaveolens</i>	137 $\pm$ 37	56 $\pm$ 6	Bd	28 $\pm$ 6	Bd	Bd	55 $\pm$ 9	31 $\pm$ 8
<i>S. cerevisiae</i>	2760 $\pm$ 374	149 $\pm$ 18	2906 $\pm$ 494	32 $\pm$ 7	Bd	Bd	Bd	16 $\pm$ 0.2

Yeast cells were grown on YNB–glucose (20 g/l) supplemented with isoleucine (1 g/l). Crude extracts were prepared as described in Materials and methods. Bd, below detection level; DC, decarboxylase; ADH, alcohol dehydrogenase; AIDH, aldehyde dehydrogenase; BCKAD, branched-chain  $\alpha$ -keto-acid dehydrogenase; ACyD, acyl-CoA dehydrogenase; AAT, alcohol acyltransferase. Data are presented as an average of three independent biological assays  $\pm$  SD.

pathway (aldehyde reduced into alcohol) instead of the acid transformation. Altogether, these data are in agreement with an Ehrlich pathway that is apparently more effective in *S. cerevisiae* than in *S. suaveolens*, and accounted for the production of 2-methylbutanol, 3-methylbutanol and 2-phenylethanol in *S. cerevisiae* (see Table 1).

In contrast, an acyl-CoA dehydrogenase (ACyD) activity was measured in the crude extract from *S. suaveolens*, while the activity of this enzyme was below detection levels in the *S. cerevisiae* extract. These data are therefore in agreement with the existence of a  $\beta$ -oxidation of isoleucine in *S. suaveolens*, and the failure to detect any ACyD activity in *S. cerevisiae* confirmed that this  $\beta$ -oxidation pathway is absent in this yeast species. However, we were unable to detect a branched-chain  $\alpha$ -keto-acid dehydrogenase (BCKAD) activity in *S. suaveolens* extract under our experimental conditions. We hypothesized that this enzyme is present in the cells but likely very unstable, since all attempts to detect any activity of this enzyme using different buffer conditions have failed.

Taken together, our phenotypic and enzymatic data clearly showed that this yeast species can catabolize branched-chain amino acids through classical Ehrlich pathway, as in *S. cerevisiae*, and through  $\beta$ -oxidation. This  $\beta$ -oxidation leads to accumulation of tiglyl-CoA, probably because of the weak activity of enoyl-CoA hydratase. This intermediate is then taken over by an alcohol acyl transferase (AAT), whose activity in *S. suaveolens* was found to be at least two-fold higher than in *S. cerevisiae*. This higher AAT could also explain that *S. suaveolens* has a stronger capacity to produce different esters as compared to *S. cerevisiae* (28 esters were detected in *S. suaveolens* vs 16 in *S. cerevisiae*). Definitive proof for the existence of a mitochondrial  $\beta$ -oxidation pathway in *S. suaveolens* will require additional work, such as using  $^{13}\text{C}$ -labelling to trace the metabolic fate of branched-chain amino acids, or cloning of the genes encoding this pathway, as done for some filamentous fungi (Maggio-Hall and Keller, 2004; Maggio-Hall *et al.*, 2008). These studies will likely help us to design process fermentation and genetic strategies to optimize the production of this biotechnologically relevant product, in order to favour the flux of isoleucine into  $\beta$ -oxidation and increase the availability of tiglyl-CoA for the AAT, instead of being diverted into the TCA.

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