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Production of sulfur compounds by several yeasts of technological interest for cheese ripening

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Abstract

Several yeasts, *Geotrichum candidum*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, *Debaryomyces hansenii*, and *Saccharomyces cerevisiae*, were studied for their ability to generate volatile sulfur compounds. These yeasts were cultivated on a synthetic culture medium supplemented with a biosynthetic precursor. With *S*-methylmethionine, dimethylsulfide (DMS) was the major sulfur compound produced. L-methionine promoted the synthesis of a wider spectrum of volatile sulfur compounds, methanethiol (MTL), DMS, dimethyldisulfide (DMDS), dimethyltrisulfide (DMTS) and *S*-methylthioacetate (MTA). Enzymatic activities possibly involved in sulfur compound synthesis were also investigated. Important L-methionine-transaminase activities, and also L-methionine-demethiolase activities were detected in cellular extracts. Their possible role in the generation of sulfur compounds and related biosynthetic pathways are also discussed.

Keywords: Yeasts; Cheese ripening; Volatile sulfur compounds; Enzymatic activities

1. Introduction

Sulfur compounds are present in many foods (Mussinan & Keelan, 1994) and beverages (Dercksen, Meijering, & Axcell, 1992; Mestres, Busto, & Guasch, 1998) and are found commonly in a range of dairy products including yoghurt (Imhof & Bosset, 1994; Ott, Fay, & Chaintreau, 1997) and ripened cheeses (Cuer, Dauphin, Kergomard, Roger, Dumont, & Adda, 1979; Grill, Patton, & Cone, 1966). In the latter case, it has been shown that the sulfur flavors comprise a structurally diverse class of molecules which provides a whole range of characteristic aromatic notes (e.g. "cheesy" and "garlic") in a particular cheese, as evidenced from the analysis of Cheddar (Manning, 1974; Urbach, 1993), Limburger (Parliment, Kolor, & Rizzo, 1982), Camembert (Dumont, Roger, & Adda, 1976; Kubickova & Grosch, 1998b) and other mold-ripened varieties (Molimard & Spinnler, 1996). Additionally, the sensory properties of these sulfur compounds are pronounced at very low concentrations due to their low odor thresholds (Kubickova & Grosch, 1998a; Cuer, Dauphin, Kergomard, Dumont, & Adda, 1979).

The origin of many sulfur flavors in cheese is associated with the production of methanethiol (MTL) not only by bacteria (Hemme & Richard, 1986; Kim & Olson, 1989; Sharpe, Law, Phillips, & Pitcher, 1977) but also by yeasts (Berger, Khan, Molimard, Martin, & Spinnler, 1999), both microbial families being used in the preparation of cheese. Numerous bacteria such as *Lactobacilli, Lactococci* (Dias & Weimer, 1998b) and *Brevibacterium linens* (Dias & Weimer, 1998a, b; Ferchichi, Hemme, Nardi, & Pamboukdjian, 1985) and the yeast *Geotrichum candidum* produce useful quantities of this compound. MTL is generally believed to result from the degradation of L-methionine by a one-step degradation pathway catalyzed by a versatile pyridoxal 5′-phosphate (PLP) dependent intracellular L-methionine-γ-lyase also called L-methionine-γ-demethiolase. This enzyme has been isolated and characterized in *B. linens* (Collin & Law, 1989; Dias & Weimer, 1998a), but it is probably present in other ripening micro-organisms (Berger et al., 1999). Another possible metabolic sequence leading to the formation of MTL from L-methionine is a two-step degradation pathway initiated by an aminotransferase also named transaminase. This

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enzyme requires the presence of an amino acceptor (e.g. α-ketoglutarate). It gives α-keto γ-methyl thio-oxobutyric acid (KMBA) as the first biotransformation product, the latter being converted to MTL most probably by a γ-demethiolase. This two-step metabolic sequence has been demonstrated in lactococci (Gao, Mooberry, & Steele, 1998) by ^{13}C nuclear magnetic resonance (NMR) performed with [^{13}C] methionine. An aminotransferase has been identified in the lactic acid bacterium *Lactococcus lactis* (Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997). This is a PLP-dependent enzyme which can catalyze the transamination of L-methionine to KMBA. Another possible two-step mechanism for the L-methionine to MTL conversion, is the oxidative deamination of L-methionine to KMBA and ammonia, KMBA being in turn converted to MTL. The oxidative deamination of sulfur amino acids including L-methionine has been demonstrated with an L-amino acid oxidase from *Proteus rettgeri* (Chen, Hudspeth Walgate, & Duerre, 1971). Further metabolism of MTL leads to the generation of a range of sulfur compounds which contribute significantly to the aroma of cheese, including dimethyldisulfide (DMDS) (Kubickova & Grosch, 1998b; Molimard & Spinnler, 1996), dimethyltrisulfide (DMTS) (Engels & Visser, 1994) by auto-oxidation of MTL (Chin & Lindsay, 1994), and also thioesters such as *S*-methyl thioacetate (MTA) and *S*-methyl thiobutyrate (MTB) (Cuer et al., 1979; Lamberet, Auberger, & Bergère, 1997a, b; Urbach, 1993).

Another important volatile sulfur compound found in cheese and beverages is dimethylsulfide (DMS). In beer, it is considered to be a typical aroma compound (Dethier, De Jaeger, Barszczak, & Dufour, 1991). Two precursors were evidenced: (i) *S*-methylmethionine through spontaneous degradation and (ii) DMSO naturally present in beer wort which is easily reduced to DMS. DMS is also produced by the cheese ripening micro-organism *G. candidum* (Berger et al., 1999) but its origin remains unknown.

In association with bacteria, yeasts are commonly present in cheese ripening cultures used in the dairy industry including *G. candidum* which develops very early during the ripening process (Bartschi, Berthier, & Valla, 1994; Lecocq, Gueguen, & Coiffier, 1996) and *Debaryomyces hansenii* (Leclercq-Perlat, Oumer, Bergère, Spinnler, & Corrieu, 1999). Although many reactions change the odor and flavor profile during the following steps, the actions of the yeasts have a definite effect on the sensory properties of the final product in model cheese media (Martin et al., 1999) or in cheeses (Molimard, Lesschaeve, Issanchou, Brousse, & Spinnler, 1997). Based on our recently published results on *G. candidum* strains (Berger et al., 1999), the aim of this work was to investigate the ability of yeasts of technological interest for cheese ripening to produce volatile sulfur compounds from L-methionine and *S*-methylmethionine. The enzymatic equipments of these micro-organisms were compared.

2. Materials and methods

2.1. Materials

MTL used as sodium methanethiolate, DMS, DMDS, MTB, *S*-methylmethionine used as DL-methionine-*S*-methylsulfonium chloride (racemic vitamin U) and L-methionine were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). MTA was obtained from Lancaster (Bischeim, France) and DMTS from Acros Organics (Noisy-le Grand, France). Noncommercially available *S*-methyl thioesters (*S*-methyl thiopropionate (MTP), *S*-methyl thio*iso*butanoate (MTIB), *S*-methyl thio*iso*valerate (MTIV) and *S*-methyl thiohexanoate (MTH)) were synthesized as previously described by Berger et al. (1999).

2.2. Micro-organisms

Ten strains of *Geotrichum candidum*, G1, G2, G3, G4, G5, G6 (Gc), G7, G8, G9 and G10 (SKW, la Ferté-sous-Jouarre, France) were used. The other yeasts employed were *Yarrowia lipolytica* 370 (Yl), *Kluyveromyces lactis* 44_8 (Kl), *Debaryomyces hansenii* 304 (Dh) from our culture collection and *Saccharomyces cerevisiae* type II (Sc) from Sigma-Aldrich (St. Quentin Fallavier, France). Yeasts strains were kept in 5% glycerol nonfat dry milk at −80°C until used.

2.3. Growth and culture conditions

Unless stated otherwise, all yeasts were grown in the dark in potato dextrose broth (PDB, Difco, Detroit, USA), pH 5.2. A preculture was incubated for 48 h at 25°C under aerobic condition (170 rpm). This preculture was used to inoculate (1%, vol/vol) the PDB culture medium, sometimes supplemented with L-methionine ($1\,g\,L^{-1}$) (+Meth) or *S*-methylmethionine ($1\,g\,L^{-1}$) (+VitU). Cultures were incubated in the dark under aerobic condition (170 rpm) during 48 h at 25°C. This time of harvest corresponds to the maximum growth for the yeasts. The purity of each culture was confirmed by testing with respect to: (i) total flora (Plate Count Agar, Biokar Diagnostics, Beauvais, France); (ii) yeast and mould (Chloramphenicol Glucose Agar, Biokar Diagnostics, Beauvais, France); and (iii) bacteria (Brain Heart Infusion, Biokar Diagnostics, Beauvais, France). Only cultures which were shown to be free from microbial contamination by plate testing were further analyzed.

2.4. Dynamic headspace extraction and GC-MS analysis of the cultures

The volatile compounds in each culture sample were extracted using a dynamic headspace analyzer (Purge and trap concentrator, HP 7695A, Hewlett Packard, Avondale, PA). Each sample tube was connected to the apparatus and heated at 60°C for 10 min and then purged with high purity helium at a flow rate of 30 mL min^{-1} for 15 min. The volatiles were extracted by adsorption on a porous polymer adsorbent Tenax trap column (60–80 mesh, 0.25 g, $30 \times 0.32\,cm^2$, Teckmar Inc., Cincinnati) at room temperature. This column was heated at 225°C for 2 min to desorb the volatiles which were directly transferred at 150°C to the head of a capillary column with cryofocusing at −150°C. Water was removed by condensation with an in-line moisture control system.

The condensed volatile compounds were analyzed by GC (HP 6890, Hewlett Packard, Avondale, PA) by heating the interface to 180°C for 1 min and automatic (splitless) injection into a nonpolar capillary column (HP-5MS, 30.0 m × 0.25 mm, 0.25 μm film thickness) at a helium flow rate of 1.6 mL min^{-1}. The oven temperature was maintained at 5°C for 8 min and then programmed from 5°C to 20°C at 3°C min^{-1} followed by a gradient of 10°C min^{-1} to a final temperature of 150°C. The GC column was also connected directly to a mass sensitive detector (HP 6890A quadrupole mass spectrometer, Hewlett Packard, Avondale, PA). The electron impact energy was set at 70 eV and data were collected in the range of 29–300 amu at a scan rate of 1.68 scans s^{-1}. Using these experimental procedures, the minimum detection limit for each sulfur compound investigated was 1 μg kg^{-1}.

2.5. Identification and quantification of sulfur compounds produced by the cultures

MTP, MTIB, MTIV and MTH were synthesized and characterized as previously described (Berger et al., 1999) and all other compounds investigated were purchased from commercial sources. Samples were kept on ice then frozen until analyzed in order to reduce auto-oxidation of MTL to a minimum. The sulfur compounds produced in the cultures were identified by GC comparison with pure references (retention time and mass spectra) and the concentration of products formed was determined using calibrated standards. Known amounts of reference compounds were added to water at varying final concentration (0, 0.05, 0.10, 0.25, 0.50 mg kg^{-1}) and for each of the five concentrations, triplicate samples were prepared. Each of these was submitted to headspace analysis three times. A linear regression between compound areas (obtained by integration of the mass sensitive detector signal) and their known concentrations (mg kg^{-1}) was performed to accurately determine the concentration of sulfur compounds in each culture.

2.6. Obtention of cell extracts

The yeasts strains were cultured at 25°C in the dark on a liquid medium (PDB, Difco) sometimes supplemented with L-methionine (1 g L^{-1}) at 200 rpm for 30 h with shaking. Cells were harvested by vacuum filtration and washed twice with 50 mL of Tris-HCl buffer 100 mM, pH 8.0. Harvested cells were disrupted by mixing with glass beads (0.5 mm ∅) in a blender (Ozyme, Bio101) at maximal speed. After centrifuging, the supernatant was considered to be the cellular extract (CE) and used for enzymatic measurements. Protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

2.7. Enzymatic activity measurements

2.7.1. Demethiolase activity

The demethiolase activity of each micro-organism was determined as described by Ferchichi et al. (1985). MTL produced from L-methionine or α-keto γ-methyl thiobutyric acid (KMBA) was reacted with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, Sigma-Aldrich, St Quentin Fallavier, France) to produce the thionitrobenzoic acid that can be detected spectrophotometrically at 412 nm. A standard curve was obtained with different concentrations of sodium methanethiolate (Fluka, St Quentin Fallavier, France). The assay mixture contained 50 mM Tris-HCl (pH 8), 0.05 mM PLP, 0.5 mM DTNB, 5 mM of L-methionine or 5 mM KMBA as the substrate, and the CE. Samples were incubated at 30°C for 1 h. The total amount of MTL formed was evaluated by measuring the variation of $OD_{412\,nm}$ from time 0 to 1 h and the rate of MTL production was calculated. Controls without CE were included and any MTL that spontaneously formed from the substrate degradation (L-methionine or KMBA) was calculated, and subtracted from the total amount of MTL produced with cells or CE. Up to 10% of total MTL produced were spontaneously formed from KMBA, while this proportion remained marginal (<1%) when L-methionine was used as the substrate. Specific demethiolase activity was expressed in nmol MTL formed g^{-1} protein s^{-1}.

2.7.2. Transaminase activity

In the presence of an amino-acid acceptor L-methionine transamination leads to the formation of its corresponding keto-acid (KMBA) and glutamate. Quantitative determination of glutamate formed during this reaction was performed with the colorimetric L-glutamic acid assay of Boehringer (Mannheim, Germany). Transaminase activity was measured as

previously described (Yvon et al., 1997) except that L-methionine was used as the substrate. Specific transaminase activity was expressed in nmol glutamate formed g^{-1} protein s^{-1}.

2.7.3. Deaminase activity

Deaminase activity was evaluated by measuring the release of ammonia from L-methionine. The assay mixture contained 50 mM Tris-HCl (pH 8), 5 mM L-methionine as the substrate, and the CE. Samples were incubated at 30°C and sampled every 10 min (during a 1 h period) to measure ammonia release. The amount of ammonia formed was evaluated using Nessler's reagent (Prolabo, France). Controls without CE were included and any ammonia spontaneously formed from the substrate degradation was calculated and subtracted from the total amount of ammonia produced with CE. Specific deaminase activity was expressed in nmol ammonia formed g^{-1} protein s^{-1}.

3. Results and discussion

3.1. Production of sulfur compounds by several cheese ripening yeasts

In our previously published results (Berger et al., 1999), the production of volatile sulfur compounds was investigated in ten strains of *Geotrichum candidum* cultivated on a liquid cheese model medium. Among these ten strains, four of them which proved to be most efficient for the production of sulfides were also capable of generating six *S*-methyl thioesters, i.e. MTA, MTP, MTB, MTIB, MTIV and MTH. The strain G6, in particular, produced these compounds in relatively large quantities but the thioesters were produced at a (10–40)-fold lower concentration than the sulfides.

From the above results, strain G6 was selected among the *G. candidum* strains tested in model liquid cheese medium. This strain as well as four other potent cheese ripening yeasts, *Y. lipolytica*, *K. lactis*, *D. hansenii*, *S. cerevisiae*, were compared for their ability to produce volatile sulfur compounds in a liquid PDB medium in the absence or in the presence of the precursors L-methionine or *S*-methylmethionine (Table 1). With the exception of the noninoculated medium, in which 16 μg kg^{-1} of DMDS were measured, only trace amounts of MTL, DMS, DMDS, DMTS and MTA were detected when yeasts were cultivated on the PDB medium.

In contrast, when the PDB medium was supplemented with *S*-methylmethionine (*S*-methylmethionine), DMS was the major volatile sulfur compound produced. In noninoculated cultures, consistent levels of DMS (1460 μg kg^{-1}) were detected, at higher levels than with yeasts *S. cerevisiae* and *K. lactis*. This clearly indicates a spontaneous degradation of *S*-methylmethionine to DMS. For the yeasts *G. candidum* and *Y. lipolytica*, the production of DMS, 9898 and 6818 μg kg^{-1} respectively, was much higher than with noninoculated medium (Table 1). This demonstrates an enzymatic biosynthesis of DMS, most probably by a demethiolating activity, from *S*-methylmethionine by these micro-organisms. DMS production was not significantly increased with *D. hansenii* in comparison with noninoculated cultures. These data strongly suggest that *S*-methylmethionine could be a possible precursor for DMS in the model liquid cheese medium (Berger et al., 1999) through a spontaneous or an enzymatic mechanism or both. However, the presence of *S*-methylmethionine in cheese remains to be elucidated.

When L-methionine was added to the culture, the volatile sulfur compounds production patterns varied greatly. Although produced in different amounts, all strains produced MTL, DMDS, DMTS and MTA, while DMS was only produced by *G. candidum* cultures (426 μg kg^{-1}). MTL was best produced by *G. candidum* and *Y. lipolytica*, highest DMDS and DMTS yields being also obtained with those cultures. This confirms the requirement for MTL as precursor for DMDS and DMTS synthesis. MTA production was, by far, highest with *G. candidum* (2900 μg kg^{-1}), whereas *S. cerevisiae* and *K. lactis* only produced 120 and 70 μg kg^{-1} of this metabolite. MTA precursors most probably are MTL (resulting from L-methionine degradation) and acetyl-CoA (resulting from glycolysis) (Yoshioka & Hashimoto, 1982, 1984). For the yeasts tested, *G. candidum* produced the widest variety and the greatest quantities of volatile sulfur compounds (Table 1).

3.2. Enzymatic activities of cellular extracts of cheese ripening yeasts

Several enzymatic activities possibly involved in sulfur compounds synthesis from the precursor L-methionine, most commonly found in milk, were searched for in the five yeasts studied. In order to evidence a possible effect of the precursor, these activities were measured in cellular extracts of yeasts cultivated in a PDB medium either supplemented or not with L-methionine (Table 2). No deaminase activity was found in any of the yeasts. Demethiolase activity, measured from the substrate L-methionine, was detected in all strains. With the notable exception of *D. hansenii*, this activity was found to be enhanced in cultures supplemented with L-methionine which indicates a possible inductive effect of the substrate on this activity. Highest demethiolase activities were obtained with *S. cerevisiae* (250 nmol g^{-1} s^{-1}) and *G. candidum* (210 nmol g^{-1} s^{-1}) in the PDB + Meth medium, and with *D. hansenii* (184 nmol g^{-1} s^{-1}) in the

Table 1
Production of sulfur compounds in PDB liquid medium, PDB liquid medium supplemented with *S*-methylmethionine (1 g L^{-1}) (+VitU), PDB liquid medium supplemented with L-methionine (1 g L^{-1}) (+Meth) by several cheese ripening yeasts[a]

	Sulfur compound concentration (μg kg^{-1})				
Yeast strain (culture medium)	MTL	DMS	DMDS	DMTS	MTA
Gc (PDB)	tr[b]	tr	tr	tr	tr
Gc (PBD + VitU)	tr	9898 ± 1980	11 ± 2	22 ± 4	9 ± 2
Gc (PDB + Meth)	66 ± 11	426 ± 68	510 ± 82	117 ± 19	2900 ± 464
Yl (PDB)	tr	nd	tr	tr	nd
Yl (PBD + VitU)	tr	6818 ± 1364	3 ± 1	2 ± 1	nd
Yl (PDB + Meth)	81 ± 13	nd	155 ± 25	97 ± 15	3 ± 1
Kl (PDB)	nd[c]	nd	nd	nd	nd
Kl (PBD + VitU)	nd	96 ± 19	nd	nd	nd
Kl (PDB + Meth)	12 ± 2	nd	13 ± 2	tr	70 ± 10
Dh (PDB)	nd	nd	tr	nd	nd
Dh (PBD + VitU)	nd	1720 ± 300	nd	nd	nd
Dh (PDB + Meth)	9 ± 1	nd	53 ± 8	12 ± 2	20 ± 3
Sc (PDB)	nd	nd	4 ± 1	tr	nd
Sc (PBD + VitU)	nd	850 ± 170	nd	nd	nd
Sc (PDB + Meth)	3 ± 1	nd	10 ± 1	8 ± 1	120 ± 18
NI (PDB)	tr	nd	16 ± 3	tr	nd
NI (PBD + VitU)	tr	1460 ± 280	15 ± 3	tr	tr
NI (PDB + Meth)	tr	nd	16 ± 2	tr	nd

[a] Yeasts employed were *Geotrichum candidum* G6 (Gc), *Yarrowia lipolytica* 370 (Yl), *Kluyveromyces lactis* 44_8 (Kl), *Debaryomyces hansenii* 304 (Dh) and *Saccharomyces cerevisiae* (Sc). Cultures were harvested after 48 h of cultivation. Noninoculated (NI) cultures were also harvested after 48 h then analyzed in order to evaluate sulfur compounds spontaneously formed. MTL: methanethiol; DMS: dimethylsulfide; DMDS: dimethyldisulfide; DMTS: dimethyltrisulfide; MTA: methylthioacetate. All experiments were carried out in triplicates and sulfur compounds concentrations reported as means ± standard deviation.
[b] Traces.
[c] Not detected.

Table 2
Enzymatic activities of cellular extracts of 48 h old cultures of yeasts cultivated in a PDB liquid medium not supplemented or supplemented with L-methionine (1 g L^{-1})[a]

Micro-organism (culture medium)	Transaminase (nmol g^{-1} protein s^{-1})	Demethiolase (nmol g^{-1} protein s^{-1})	Deaminase (nmol g^{-1} protein s^{-1})
Gc (PDB)	583.3 ± 200	105 ± 66	nd[b]
Gc (PDB + Meth)	1738.3 ± 33.3	210 ± 41	nd
Yl (PDB)	121.7 ± 3.3	94 ± 12	nd
Yl (PDB + Meth)	287.3 ± 11.2	108 ± 12	nd
Kl (PDB)	nd	54 ± 48	nd
Kl (PDB + Meth)	nd	89 ± 7	nd
Dh (PDB)	131.7 ± 8.3	184 ± 2.5	nd
Dh (PDB + Meth)	nd	74 ± 2.5	nd
Sc (PDB)	nd	75.5 ± 2	nd
Sc (PDB + Meth)	550 ± 150	250 ± 66.5	nd

[a] *Geotrichum candidum* G6 (Gc), *Yarrowia lipolytica* 370 (Yl), *Kluyveromyces lactis* 44_8 (Kl), *Debaryomyces hansenii* 304 (Dh) and *Saccharomyces cerevisiae* (Sc). All experiments were carried out in triplicates and activities reported as means ± standard deviation.
[b] Not detected.

PDB medium. No transaminase activity was detected in *K. lactis*. Apart from *D. hansenii*, for which transaminase activity was only detected when cultivated in the PDB medium, transaminase activity was increased in cellular extracts from PDB + Meth medium (Table 2). *G. candidum* demonstrated the highest transaminase activities both in PDB medium (583 nmol g^{-1} s^{-1}) and in PDB + Meth medium (1738 nmol g^{-1} s^{-1}).

These results suggest that L-methionine degradation to MTL probably proceeds via a two-step degradation pathway initiated by a transaminase, which is probably the main route, but also through a one-step degradation pathway, through a L-methionine-γ-demethiolase pathway as described in *Brevibacterium linens* (Ferchichi et al., 1985; Dias & Weimer, 1998a). MTL can, in turn, give rise to other sulfur compounds including DMDS, DMTS (by auto-oxidation of MTL), DMS probably (by degradation of *S*-methylmethionine), and also *S*-methylthioesters.

3.3. Diversity of the intraspecific activities for ten strains of Geotrichum candidum

In order to measure the enzymatic activities possibly involved in L-methionine catabolism, ten strains of *Geotrichum candidum* were cultivated on a PDB medium enriched with L-methionine. Three types of enzymatic activities were measured in cell extracts: (i) the direct degradation of L-methionine to MTL, (ii) the degradation of L-methionine into MTL in the presence of the acceptor (α-keto-glutaric acid), and (iii) the degradation of the KMBA to MTL (Fig. 1). The direct degradation of the L-methionine to MTL changed with the strain and was between 20 and 90 nmol g^{-1} s^{-1}, strains G6, G10, G8 and G3 being the highest producers. On the contrary, the conversion of KMBA to MTL was generally much more efficient varying from 50 to 150 nmol g^{-1} s^{-1}, except for strain G3 whose MTL production was not significantly different from KMBA or L-methionine. To check for any limitation of the transamination step, possible substrate limitation of the transamination of L-methionine was examined. The acceptor α-keto glutarate (α-KG) was therefore added to the reaction assay. The addition of α-KG did not affect MTL production rates as compared to assay without added α-KG; the amine acceptor did not seem to be a limiting factor (Fig. 1). For transaminase, activities were between 1400 nmol glutamic acid g^{-1} s^{-1} and 7600 nmol glutamic acid g^{-1} s^{-1} depending on the strain considered (data not shown). These values appeared to be much higher than the demethiolation activity and did not appear to have a limiting effect. An interesting feature is that strain G3, whose γ-demethiolase activities on L-methionine and on KMBA were the same, was also the one which exhibited the highest transaminase activity (all other strains had similar transaminase activities). Perhaps a much higher transaminase activity is necessary to accumulate KMBA in an amount sufficient to achieve optimal MTL production.

The important differences between strain behaviors is a difficult problem to solve for efficient screening. However, it seems that several enzymes could be limiting for the synthesis of MTL from L-methionine. To be efficient, strain screening must be done over several enzymatic activities.

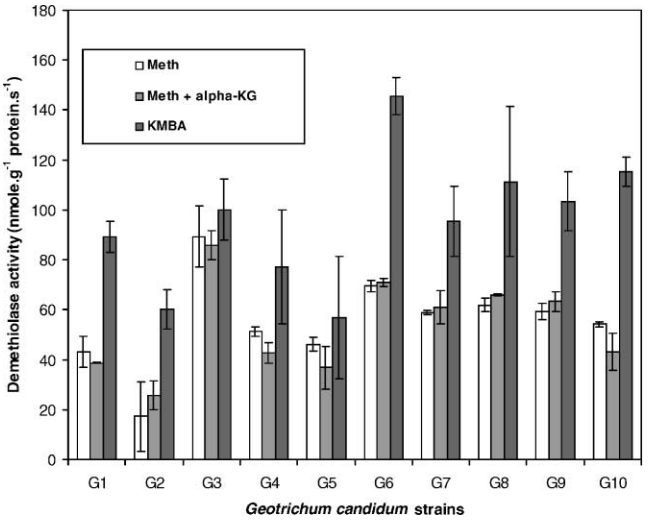

Fig. 1. Demethiolase activity measured from substrates L-methionine (Meth) or α-keto γ-methyl thio-butyric acid (KMBA) in cellular extracts of ten *Geotrichum candidum* strains. Cultures were carried out in a PDB liquid medium supplemented with L-methionine (1 g L^{-1}) and harvested after 48 h of cultivation. All experiments were carried out in triplicates and activities reported as means $\pm$ standard deviation.

4. Conclusions

Yeasts have interesting capabilities to produce sulfur flavor compounds essential for cheese flavors. Among the yeasts commonly used in cheese making technologies *G. candidum* seems to have the highest potential, but *Y. lipolytica* is also able to produce DMS, DMDS and DMTS in quite large quantities when L-methionine is provided. L-methionine added to a standard medium generally enhanced sulfur compound production, except in the case of *D. hansenii*. A two step pathway using: (i) a transaminase which produces the KMBA from L-methionine, and (ii) a degradation of the KMBA produced into MTL, seems an alternative degradation route except for *K. lactis,* for which transaminase activity was not detected. In this last species (or strain) the volatile sulfur compounds produced may be provided by the attack of L-methionine via a L-methionine-γ-lyase as in *B. linens* (Dias & Weimer, 1998a).

G. candidum is more active on KMBA than on L-methionine. We can therefore suspect a limitation of the transaminase activity in this micro-organism. Our results suggest that a two-step pathway can operate in *G. candidum* but concomitant activity of the one-step pathway may be possible. One important final point is the wide inter-strain variability which hinders setting up fast screening methods.

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