Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 338 (2005) 299–305
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Development and validation of a plate technique for screening of microorganisms that produce volatile sulfur compounds

Hugues Guichard[a], Pascal Bonnarme[b,*]

[a] *ADRIA Normandie, Boulevard du 13 juin 1944, BP2, F-14310 Villers Bocage, France*
[b] *Institut National de la Recherche Agronomique, UMR Génie et Microbiologie des Procédés Alimentaires, F-78850 Thiverval-Grignon, France*

Received 24 September 2004
Available online 25 January 2005

Abstract

Volatile sulfur compounds (VSCs) are of major importance for flavor development in foodstuffs such as cheeses. Such compounds originate from the amino acid L-methionine, which can be degraded to methanethiol (MTL), a common precursor to a variety of other VSCs. A plate assay based on double-layer petri dishes containing 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), a chemical used for the estimation of free thiols, in the upper layer provides an easy and reliable detection method for thiol-producing, cheese-ripening microorganisms. MTL production was quantitated by measuring the yellow–orange color intensity resulting from reaction with DTNB. Using this method, 18 *Geotrichum candidum* strains isolated from cheeses were compared, and the color intensity was found to be correlated with the production of microbial VSCs as measured by gas chromatographic analysis.

Keywords: Plate assay; Volatile sulfur compounds; Screening; *Geotrichum candidum*

Volatile sulfur compounds (VSCs)[1] are generally present in minute amounts in ripened cheeses. However, owing to their low detection threshold, VSCs play a major role in cheese flavoring [1,2]. Sulfur flavors encompass a structurally diverse class of molecules that provide a range of characteristic aromas. They are involved in the basic "cheesy" flavor but can also generate specific flavors (e.g., cabbage, garlic), as shown by the analyses of cheddar [3,4], Limburger [5], Camembert [1,6,7], and other mold-ripened varieties of cheese [8]. Therefore, the occurrence of such compounds in cheeses makes a significant contribution to their distinctive aroma.

Most VSCs, such as dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) [9,10], 2,4-dithiapentane (DTP) [6], and *S*-methylthioesters [2,11], share a common precursor, methanethiol (MTL), which arises from the degradation of the sulfur–carbon bond of L-methionine. MTL production can be catalyzed either by bacteria or by yeasts [12], with both microbial communities being part of the cheese ecosystem. Therefore, the degradation pathways of L-methionine to MTL in such microorganisms have been studied [13]. Among yeasts, *Geotrichum candidum*, which is essential for the ripening of mold-ripened cheeses such as Camembert and Livarot [14], has been shown to produce consistent amounts of MTL together with a wide variety of other VSCs [15,16]. Because of the importance of MTL in cheese flavoring, as well as the high volatility and reactivity of this compound [10], it is essential to be able to compare cheese-ripening strains on the basis of their ability to produce MTL.

* Corresponding author. Fax: +33 1 30 81 55 97.
E-mail address: bonnarme@grignon.inra.fr (P. Bonnarme).

[1] *Abbreviations used:* VSC, volatile sulfur compound; DMDS, dimethyl disulfide; DMTS, dimethyl trisulfide; DTP, 2,4-dithiapentane; MTL, methanethiol; DTNB, 5,5′-dithio-bis-2-nitrobenzoic acid; GC–MS, gas chromatography–mass spectrometry; YEGC, yeast extract glucose chloramphenicol agar; BHI, brain heart infusion agar; DMS, dimethyl sulfide; MTA, *S*-methyl thioacetate; MTB, *S*-methyl thiobutyrate.

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2004.12.027

The main objective of this work was to develop a simple and reliable screening method for the determination and comparison of in situ thiol-producing abilities of a population of *G. candidum* strains isolated from mold-ripened cheeses. A plate colorimetric method based on the reaction of 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent) with free thiols was developed on double-layer petri dishes and subsequently used to compare the ability of 18 strains to produce MTL. Color measurements were correlated with the total production of VSCs quantified by the purge and trap gas chromatography–mass spectrometry (GC–MS) technique. The plate technique could, therefore, be easily employed for screening of thiols other than MTL or of VSC-producing microorganisms.

Materials and methods

Microbial strains and culture conditions

With the exception of GcG (Degussa, La Ferté-sous-Jouarre, France), all of the *G. candidum* strains studied were originally isolated from mold-ripened cheeses—Camembert, Livarot, Pont l'Evêque—of the French Normandy (UCMA collection of Laboratoire de Microbiologie Alimentaire, University of Caen, France). They were grown in a previously described culture liquid medium [16] that was incubated for 48–72 h under aerobic conditions and agitation (150 rpm, 5-cm diameter stroke). Cultures were stored as aliquots in nonfat dry milk with 5% glycerol at −80 °C until used. The purity and numeration of each aliquot were confirmed by plating yeasts and molds on yeast extract glucose chloramphenicol agar (YEGC, Biokar Diagnostics, Beauvais, France) and plating bacteria on brain heart infusion agar (BHI, Biokar Diagnostics).

Screening procedure

Double-layer petri dishes were made from two media: medium A (layer A) and medium B (layer B) (Fig. 1). Medium A was prepared as follows. A concentrated solution composed of agar (8.4% w/w) and NaCl (12% w/w) was sterilized (120 °C for 20 min) and maintained in a liquid state at 80 °C until used. A cheese slurry was also prepared. Cheese curds were thawed (4 °C overnight) and mixed (60% w/w) with sterile water using a laboratory blender (Waring, Fisher Labosi, Elancourt, France), the pH was adjusted to 5.0 if necessary, and the slurry was sterilized at 100 °C for 30 min. Once sterilized, 85% (v/v) of the cheese slurry was mixed to 15% (v/v) of the concentrated solution. The mixture, called medium A, was homogenized at 25,000 rpm for 2 min (Ultra-Turrax, VWR International, Fontenay-sous-Bois, France). When the temperature reached approximately 50 °C, it was inoculated with the desired inoculum concentration (10^4 cfu/g of medium A), and then rapidly poured into plates (20 ml/90-mm diameter plate). Medium B was prepared as follows. Stock solutions of agar (36 g/L) and DTNB (4 mM) were sterilized at 120 °C for 20 min and at 110 °C for 15 min, respectively. Medium B was prepared by mixing 67% (v/v) of the agar solution to 33% (v/v) of the DTNB stock solution and was maintained at 60 °C until used. Medium B (10 ml) was gently poured on top of layer A (Fig. 1). The plates were subsequently incubated at 14 °C (cheese-ripening temperature), and color measurements were performed when inoculated (day 0), after 1 day (day 1), and then every day after the fourth day (day 4).

Color measurements

Color intensity measurements were carried out with a CM-2002 spectrocolorimeter (Minolta, Carrières sur Seine, France) driven by SpectraMagic 1.01 software (Minolta), as described previously [17]. In the $L \times a \times b$ three-dimensional response system, the b color value was the best estimate for yellow color intensity measurements. Color measurement was analyzed by taking three independent measurements in a single session at the bottom of each plate (Fig. 1). No effect of pH on color intensity was noticed over a pH range of 6–8 for different MTL concentrations (0–100 μM). This indicates that

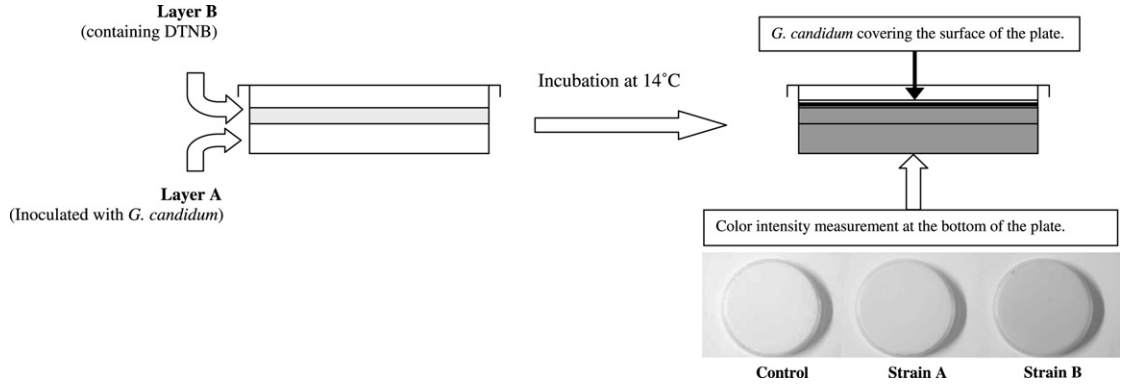

Fig. 1. Detection of thiol-producing microorganisms in double-layer agar plates.