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Evaluation of a quantitative screening method for hydrogen sulfide production by cheese-ripening microorganisms: The first step towards L-cysteine catabolism

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Abstract

A practical adaptation of the methylene blue reaction for hydrogen sulfide quantification was developed to perform microbial selection. Closed plate flasks containing a zinc-agar layer above the liquid microbial culture are proposed as a trap system where the H_2S can be retained and then quantified by the methylene blue reaction. Using this quantitative method, the ability to produce H_2S was studied in several cheese-ripening microorganisms. Our aim was to select strains that produce the highest quantities of H_2S as the main product of L-cysteine catabolism. Thirty seven yeast and bacteria strains were cultivated with or without L-cysteine. The separation between the growth medium and the H_2S trapping layer displayed good performance: all the studied strains grew efficiently and only negligible loss of H_2S was observed during culturing. The strains displayed large differences in their H_2S production capabilities: yeast strains were greater producers of H_2S than bacteria with production strain-related in both cases. Furthermore, the relationship between H_2S production and L-cysteine consumption was analyzed, which made it possible for us to select microorganisms with high capacity in L-cysteine degradation. The production of volatile sulfur compounds was also studied and the possible effect of culture pH and metabolic differences between strains are discussed.

Keyword: L-cysteine; Hydrogen sulfide; Volatile sulfur compounds; Screening; Cheese-ripening microorganisms

1. Introduction

Volatile sulfur compounds (VSC) are major flavoring compounds in cheeses and many other fermented foods. They originate mainly from L-methionine catabolism which has been extensively studied in cheese-ripening microorganisms including yeasts (Arfi et al., 2002; Bondar et al., 2005) and bacteria (Bonnarme et al., 2000; Amarita et al., 2004). L-cysteine has a lower concentration than L-methionine in cheese curd (five times lower (Wood et al., 1985)) and probably for this reason has been rather neglected up to now in VSC generation studies. The role of L-cysteine as a potent VSC precursor is, however, well established in some fermented beverages (Hansen and Kielland-Brandt, 1996; Duan et al., 2004; Marchand et al., 2002; Sutherland et al., 2003). In beer or wine production, the excess of L-cysteine in the initial medium leads to increased H_2S formation during fermentation through an L-cysteine desulfhydration mechanism (Lawrence and Cole, 1968). Furthermore, enzymes involved in L-cysteine catabolism have been described in bacteria (Yoshida et al., 2002; Aubel et al., 2002; Auger et al., 2005) with the recurrent production of H_2S.

According to these observations, we decided to develop a screening methodology to evaluate H_2S production from L-cysteine in 37 cheese-ripening microorganisms, including 20 yeast and 17 bacterial strains.

Hydrogen sulfide (H_2S) determination has been done through a large panel of methods, including electrochemical, chromatographic and spectroscopic procedures. Among the spectroscopic detections, methylene blue reaction is the most common approach for H_2S analysis and a number of recent developments

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Table 1
Yeast and bacterial strains tested in this study

Strains	Reference numbers
Yeasts	
Debaryomyces hansenii (DH)	DH47(8), DH142, DH304
Geotrichum candidum (GC)	GC108[a], GC103[a], GC77[a], GCD, GCG[b]
Kluyveromyces lactis (KL)	KL44(8), KL640[c], KL643[c], KLC15, KLC22
Saccharomyces cerivisiae (SC)	SC42, SC45(3)
Yarrowia lypolytica (YL)	YL200, YL274, YL370, YL136463[c], YLW29[c]
Bacteria	
Arthrobacter spp (AR)	AR7(2), AR225
Arthrobacter arilaitensis (AR)	AR269, AR175[d]
Brevibacterium linens (BL)	BL918[d], BL1939[e], BL 9175[e], BLC308, BLC206, BLC212
Corynebacterium glutamicum (CO)	COD13
Corynebacterium spp (CO)	COC45, COC55, COC74
Micrococcus luteus (ML)	ML790
Staphylococcus equorum (SE)	SE1265
Staphylococcus xylosus (SX)	SXJ870[d]

[a] LMA: Laboratoire de Microbiologie Appliquée, Caen, France.
[b] Commercial strain (Degussa, La Ferté-sous-Jouarre, France).
[c] CLIB: "Collection de Levures d'Intérêt Biotechnologique", Unité de Microbiologie et Génétique. Moléculaire (INRA-CNRS-INA-PG), Grignon, France.
[d] CNRZ: Centre National de Recherche en Zootechnie, collection de bactéries laitières d'intérêt technologique, INRA Jouy en Josas, France.
[e] ATTC: American Type Culture Collection, LGC Promochem Sarl, Molsheim, France.

have led to substantial improvements in sensitivity, reliability and cheapness (to review see Lawrence et al., 2000).

Few methods have been developed for the direct determination of H_2S production by microorganisms. Due to its high volatility, H_2S is generally trapped in alkaline solutions (pH > 10) which can prevent the growth of microorganisms. Consequently, the methods developed usually keep the culture media and trapping solution separated. In this respect, mini-bioreactor-gas collector (Garcia et al., 2003) plates overlaid by specific membrane (Duan et al., 2004) flow analysis combined with spectrophotometric detection (Yuan and Kuriyama, 2000) or a detection tube method (Park and Kim, 2004) have been developed as analytical methods. Nevertheless, no such approach has been used for screening purposes due to complexity and/or expense, even though these methods share good specificity, sensitivity and reliability. Furthermore, in conventional tests for microbial identification where selective media for H_2S detection has been formulated, for example in *Salmonella* spp. detection (Shelef and Tan, 1998), no precise quantification of H_2S has been achieved even though high sensitivity has been obtained.

To evaluate H_2S production from L-cysteine by cheese-ripening microorganisms, a closed system based on an original double layer method was therefore developed and H_2S was quantified *in situ* using the methylene blue reaction. An initial approach to VSC generation from L-cysteine by the most efficient H_2S-producing stains was also carried out.

2. Materials and methods

2.1. Microorganisms and culture conditions

Twenty yeast and seventeen bacterial strains used in the study (Table 1) were originally isolated from cheeses and belong to our laboratory's own collection (unless otherwise indicated). The microorganisms were stored in Difco-skim milk (BD-Difco, Le Pont de Claix, France) containing 10% glycerol at −80 °C. Yeasts were grown in potato dextrose broth (PDB, BD-Difco) and bacteria in a brain heart infusion broth (BHI, Biokar Diagnostic, Beauvais, France), respectively. Preculturing was carried out in 100 ml flasks containing 20 ml of a medium PDB or BHI inoculated with 200 µl of the strain stock suspension. They were agitated (150 rpm) for 48 h at 25 °C and subsequently used for the inoculation of screening-flasks or cultures where production of VSC was analyzed. In the screening test, 5 ml of PDB or BHI supplemented or not with L-cysteine (1.0 g/l final concentration) were dispensed into the screening-flasks (see below) and inoculated with 200 µl of each preculture strain. The screening-flasks were then incubated at 25 °C and 90 rpm for 48 h. Flasks without inoculation were prepared to take into account the spontaneous degradation of L-cysteine. Each culture was made in triplicate. Apart from the determination of H_2S, the absorbance, dry weight and pH of the cultures were also determined at the beginning and after 48 h.

In addition, cultures for the analysis of VSC were prepared in triplicate: 100 ml of media in 500 ml flask were supplemented or not with L-cysteine (1.0 g l^{-1} final concentration) and inoculated with 200 µl of precultures. After 48 h incubation (150 rpm, 25 °C), 5 ml of each microbial culture were transferred into solid phase microextraction (SPME) vials at 4 °C and stored at −80 °C until analysis.

The purity of each culture was checked by plating the yeasts on Yeast-chloramphenicol-dextrose agar (YGC, BD-Difco) and the bacteria on BHI with amphotericin B (Sigma-Aldrich, St Quentin Fallavier, France). Initial and residual L-cysteine was quantified using the ninhydrine acid method (Gaitonde, 1967).

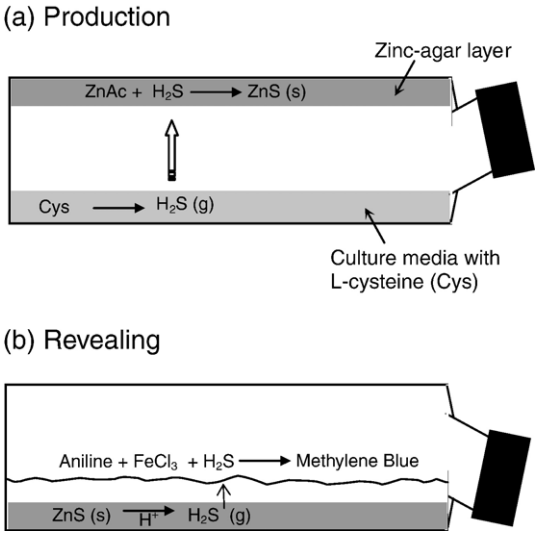

Fig. 1. Trap system for the capture of the H_2S (a) and quantification by the methylene blue synthesis reaction (b). See the text for explanation.