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Method for the rapid quantitative detection of lipolytic activity among food fermenting microorganisms

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Abstract

A standard method for the detection of free fatty acids (FFAs) in milk was modified and applied to the measurement of the lipolytic activity of microorganisms in a model system containing either homogenised pork or beef fat tissue. The increase in FFAs was measured colorimetrically using palmitic acid as a standard. Among the strains tested, two strains of *Staphylococcus xylosus* and one strain of *Staphylococcus carnosus* were found to display lipolytic activity. For all strains, a higher increase in FFA was observed in broth supplemented with pork fat than with beef fat. All three strains displayed lipolytic activity when tested on tributyrin agar plates. © 1997 Elsevier Science B.V.

Keywords: Lipolysis; *Staphylococcus*; Fat model system; Starter culture

1. Introduction

Starter cultures are commonly used for the production of fermented meat products and some are known to have the potential to enhance flavour by lipolytic activities (Miteva et al., 1986). Strains of *Staphylococcus carnosus* and *Staphylococcus xylosus* are used as commercial starter cultures for raw sausage fermentation and were found to have lipolytic activity (Molina et al., 1991). The authors determined the free fatty acids (FFAs) titrimetrically. Further screening methods for bacterial lipolytic activity have been described by Kouker and Jaeger (1986) who used a plate assay supplemented with a substrate containing a fluorescent dye, whereas Sørensen and Jakobsen (1996) examined lipase activity of cell-free extracts on *p*-nitrophenyl myristate colorimetrically. Sørensen et al. (1993) examined the ability of *Staphylococcus xylosus* to hydrolyse pork fat under conditions relevant for raw sausage fermentation and analysed FFAs by using gas chromatography. In the present study, resting cells of three *Staphylococcus* strains and three *Lactobacillus* strains were tested for their lipolytic activity on tributyrin agar plates and in a broth supplemented either with tributyrin, pork or beef fat. The FFAs were determined colorimetrically as a complex of their copper salts and sodium diethyl dithiocarbamate, which has been described as a sensitive and rapid test method for the global de-

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termination of FFAs, with the possibility of being automated (Anonymous, 1991). The method is already used for the detection of lipolysis in milk. It offers the advantage of rapid determination of FFAs without the need for expensive equipment.

2. Materials and methods

2.1. Strains and growth conditions

The strains investigated (see legend to Fig. 2) were kindly provided by Chr. Hansen (Hørsholm, Denmark). *Staphylococcus* strains were grown in a medium containing 0.1% glucose (Merck, Darmstadt, Germany), 1% tryptone (Oxoid, Hampshire, UK), 0.5% yeast extract (Oxoid) and 0.5% NaCl (Merck), pH 7.2, at 30°C. *Lactobacillus* strains (*Lb. plantarum, Lb. pentosaceus* and *Lb. pentosus*) were grown in MRS medium (Oxoid) at 30°C.

2.2. Screening for lipolytic activity using agar plates

Overnight cultures were harvested after centrifugation at 3000 rpm (relative centrifugal force (RCF) = 1680g, Hermle Z380, Wehengen, Germany) and were washed once with 0.05 M phosphate buffer (Merck), pH 7. Subsequently, the cell pellet was resuspended in 0.05 M phosphate buffer to a final optical density of ten, measured with a spectrophotometer (Shimadzu, Tokyo, Japan) at a wavelength of 578 nm. The cell suspension was concentrated to ensure the detection of even low lipolytic activities. The cell suspension (50 μl) was added to wells with a diameter of 0.7 cm within tributyrin agar base plates (Merck) that were supplemented with 1% tributyrin (Merck). Lipolytic activity was detected qualitatively by a clear zone surrounding wells in the turbid tributyrin agar. The incubation was continued for up to two weeks for detection of low lipolytic activities.

2.3. Screening for lipase activity in liquid fat model systems

Cell pellets obtained as described above (Section 2.2) were resuspended in a broth containing 0.5% peptone (Oxoid), 0.3% yeast extract (Oxoid), 0.2% NaCl (Merck), pH 7.0. This broth was supplemented either with 4% (w/v) pork fat, 4% (w/v) beef fat or 1% (w/v) tributyrin (Merck) and was homogenised using an Ultra-turrax T25 (Janke and Kunkel, IKA-Labortechnik, Staufen, Germany) and sterilized. After sterilization, the fat was re-emulsified by vigorously shaking it. The cell concentration in the model system was adjusted to $1 \cdot 10^9$ CFU/ml. Incubation was carried out at 30°C, with shaking. Control batches consisted of broth supplemented with fat but without cells. *Staphylococcus* strains were incubated for two days, whereas *Lactobacillus* strains were incubated for two weeks.

2.4. Determination of free fatty acids

A method proposed for the evaluation of free fatty acids in milk was applied (Anonymous, 1991). Free fatty acids were extracted from a sample volume of 0.5 ml by an organic solvent mixture (CHM) composed of chloroform (49%), *n*-heptane (49%) and methanol (2%). 'Copper salts' were formed by the addition of a copper reagent, consisting of a saturated solution of NaCl, which was supplemented with copper nitrate and triethanolamine. Colour development was achieved by the formation of a complex between copper salts with 0.1% sodium diethyl dithiocarbamate in *n*-butanol. The optical density was measured spectrophotometrically at 440 nm (Shimadzu). A standard curve was prepared using palmitic acid (Sigma, St. Louis, MO, USA) in a 25-fold higher concentration range (0.052–1.6 mg/ml) than that described for milk (Anonymous, 1991). If the OD_{440} of a sample was higher than two, the sample was diluted with CHM-mixture prior to the addition of sodium diethyl dithiocarbamate. A dilution of the final yellow colour complex with sodium diethyl dithiocarbamate was not found to follow a linear relationship (unpublished). Each sample was determined in duplicate.

3. Results and discussion

All three *Staphylococcus* strains displayed lipolytic activity when tested on tributyrin agar base supplemented with 1% tributyrin, whereas the *Lactobacillus* strains showed no activity, even after an incubation period of two weeks. For the quantification of free fatty acids in the fat model system, a

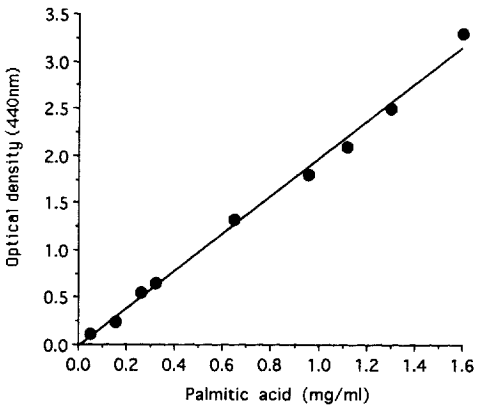

Fig. 1. Standard curve for the quantitative determination of free fatty acids in the fat model system with palmitic acid as the standard, within a concentration range of 0–1.6 mg/ml.

standard curve using palmitic acid was prepared, as shown in Fig. 1. The lipolytic activities of the three *Staphylococcus* strains (*Staphylococcus carnosus* MC-ES, *Staphylococcus xylosus* DD-34 and CM-258) tested are displayed in Fig. 2 Fig. 3. No increase in FFAs was detected in broth containing these *Staphylococcus* strains when supplemented with tributyrin, even though lipolytic activity was detected on tributyrin agar plates (results not shown). It has been suggested that the 'copper soap' method measures fatty acids, containing at least four carbon atoms, and these copper salts are preferentially soluble in the organic phase (Anonymous, 1991). Our results revealed that the method was not sensitive for the detection of butyric acid under our experimental conditions. A slight increase in FFAs was further observed in all control batches (blanks) that had been supplemented with fat, but had not been inoculated. However, the concentration of FFAs in the inoculated batches was significantly higher and increased further within two days, compared with the blanks. No increase in FFAs was observed when *Lb. plantarum, Lb. pentosaceus* and *Lb. pentosus* were incubated in the fat model systems, which was consistent with the results obtained on tributyrin agar plates. The so-called 'copper soap method' was found to be suitable for the detection and quantification of lipolytic activities of microorganisms towards pork and beef fat. These fats are components of fermented meat products and it is therefore meaningful to investigate the presence of lipolytic activity of starter cultures on natural fats prior to more extensive studies. Nielsen and Kemner (1989) examined the lipolytic activity of meat starter cultures on pork, beef and lamb fat and concluded that, even though a large variation in lipolytic activity among the examined microorganisms was evident, no special preference between the different fat types could be detected. In this investigation, however, all *Staphylococcus* strains showed higher lipolytic activities in the batches supplemented with pork fat than in those containing beef fat after two days. Nielsen and Kemner (1989) did not mention how long the

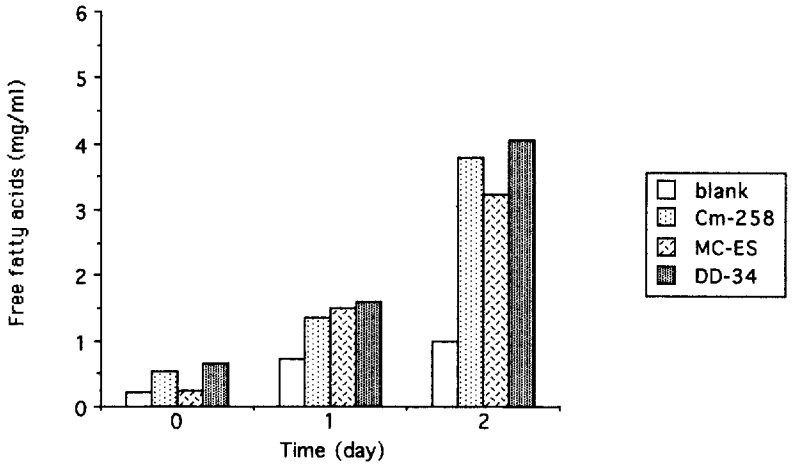

Fig. 2. Lipolytic activity in a fat model system supplemented with beef fat and stationary phase cells of *Staphylococcus xylosus* CM-258, *Staphylococcus carnosus* MC-ES and *Staphylococcus xylosus* DD-34. The blank contains fat but no cells.

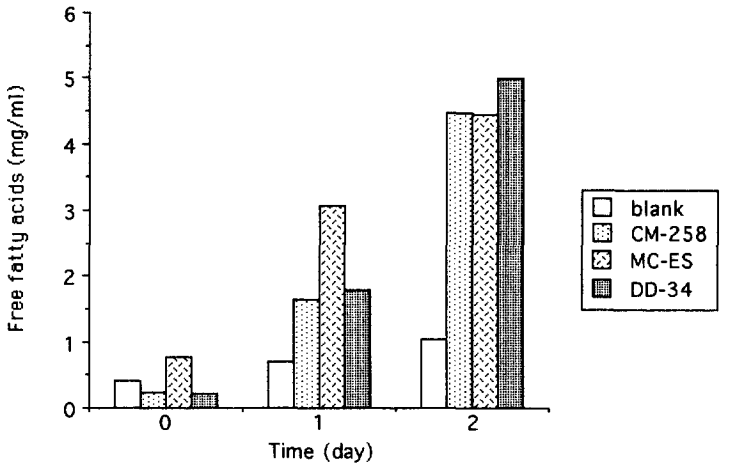

Fig. 3. Lipolytic activity in a fat model system supplemented with pork fat and stationary phase cells of *Staphylococcus xylosus* CM-258, *Staphylococcus carnosus* MC-ES and *Staphylococcus xylosus* DD-34. The blank contains fat but no cells.

stationary cells were incubated in the model system. They revealed that all strains tested displayed lipolytic activity towards the natural fats, whereas no activity was observed in some cases on agar plates supplemented with tributyrin. In their study, the authors used blanks that did not contain the natural fat. Our investigations revealed that there was an increase in FFAs in all control batches supplemented with fat, even though they did not contain resting cells. Therefore, the lipolytic activity that was detected by Nielsen and Kemner (1989) could have been due to chemical hydrolysis of fat and was not necessarily caused by microbial enzymatic activity. The results obtained in this study using the 'copper soap' method for the detection of FFAs in a pork or beef model system indicate a meaningful application of this method for the evaluation of FFAs in meat systems.

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