Original article

Purification and characterization of an extracellular esterase from *Arthrobacter nicotianae* 9458

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Abstract — A 32 kg·mol^{-1} extracellular esterase from *Arthrobacter nicotianae* 9458 was purified to homogeneity by 4 chromatographic steps. The optimum pH and temperature for enzyme activity for β-naphthyl butyrate were 7.0 and 30 °C, respectively. The esterase retained between 50–55% of the maximum activity at pH 5.5 and 15 °C and was completely inactivated by heating for 1 min at 65 °C. Among the β-naphthyl derivatives of various chain length (C2-C18:1), the highest activity was on β-naphthyl butyrate. The enzyme was moderately active on tributyrin and was less active on tricaproin. The esterase was markedly inhibited by phenylmethylsulfonyl fluoride and to a lesser extent by EDTA. Divalent cations such as Fe^{2+}, Sn^{2+}, Ca^{2+} and Hg^{2+} also inhibited the enzyme. This characterization showed that the extracellular esterase of *Arthrobacter nicotianae* 9458 may contribute to the ripening of smear surface-ripened cheeses.

Arthrobacter / esterase / smear surface-ripened cheese

Résumé — **Purification et caractérisation d'une estérase exocellulaire produite par *Arthrobacter nicotianae* 9458.** Une estérase de 32 kg·mol^{-1}, isolée d'*Arthrobacter nicotianae* 9458, a été purifiée à l'homogénéité selon 4 étapes chromatographiques. Les valeurs optimales de pH et de température pour l'activité de l'enzyme étaient respectivement de 7,0 et 30 °C. L'enzyme conservait entre 50 et 55 % de l'activité maximale respectivement à pH 5,5 et à 15 °C. Elle était complètement désactivée après un traitement à 65 °C pendant 1 min. Testée sur les dérivés β-naphthyl de différents poids moléculaires (C2 - C18:1), elle présentait l'activité la plus élevée sur le β-naphthyl butyrate ;

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elle était moyennement active sur la tributyrine et encore moins active sur la tricaproïne. L'estérase était partiellement inhibée par l'EDTA et très fortement par le phenylmethylsulfonyl fluoride. Les cations divalents comme Fe^{2+}, Sn^{2+}, Ca^{2+}, Hg^{2+} avaient également un effet inhibiteur. Il résulte de cette caractérisation que l'estérase d'*Arthrobacter nicotianae* 9458 peut contribuer à la maturation de fromage à croûte lavée.

Arthrobacter / estérase / fromage à croûte lavée

1. INTRODUCTION

Arthrobacter spp. are Gram-positive chemoorganotrophs with a respiratory metabolism; they are never fermentative, and are widely distributed among the bacterial populations in the soil. *Arthrobacter* spp. are major components of the smear microflora of surface-ripened cheeses. Bacterial smear surface-ripened cheeses (e.g., Limburger, Brick, Münster, Saint-Paulin, Appenzeller, Trappist, Taleggio and Quartirolo) can be loosely defined as cheeses in which the large numbers of bacteria present on the surface of the cheese play a significant role in determining the final characteristics and attributes of the cheese. *Arthrobacter* spp., together with yeasts and *Brevibacterium linens*, were the main microorganisms found in Limburger cheese during ripening [10]. Coryneform bacteria from 21 brick cheeses (including Limburger, Romadur, Weinkäse and Harzer) produced by 6 German dairies were identified as *Arthrobacter nicotianae*, *B. linens*, *Corynebacterium ammoniagenes* and *C. variabilis* [49]. After an initial variability of the surface microflora of Tilsiter cheeses from 14 Austrian cheese plants, the number of yeast cells decreased, followed by the growth of a mixed population composed of *A. citreus*, *A. globiformis*, *A. nicotianae*, *A. variabilis*, *B. linens* and *B. ammoniagenes* [12, 13]. The yellow-green colouration of the surface of Taleggio cheese is mainly caused by *A. globiformis* and *A. citreus* [40]. Other Italian cheeses, e.g., Quartirolo and Robiola, contained *Arthrobacter* spp. on the cheese surface [39]. *Arthrobacter* spp. are also major components of mould surface-ripened cheeses, such as Brie and Camembert [32].

Low molecular mass compounds are produced in the smear and surface layer of these cheeses by the combined action of hydrolases, mainly microbial and of extracellular origin, although intracellular and cell-bound enzymes may also contribute. The diffusion of these compounds into the interior of the cheese is required for the development of the characteristic qualities of the product. The enzymes or microbial cells are considered much too large to diffuse into the cheese [20, 34]. Free fatty acids make an important contribution to the development of the characteristic flavour during cheese ripening. Woo et al. [50] reported that the total free fatty acids in Limburger and in 2 samples of Brick cheeses ranged from 402 to 4 187 mg·kg^{-1}. The most significant factor in determining the degree of lipolysis in bacterial smear surface-ripened cheeses is the production of lipases and esterases by the surface microflora. Lipolytic and esterolytic enzymes from yeasts [1, 25, 44], filamentous fungi [42] and *Micrococcus* [2, 30] have been extensively studied, as well as lipolytic and esterolytic enzymes of *B. linens* [14, 17, 18, 29, 43, 47]. Lipolytic and esterolytic activities have also been detected in lactic acid bacteria [4, 5, 23, 26, 27, 35, 38, 41] and in *Propionibacterium* [8, 36, 37].

Despite the high cell numbers in the smear and their importance in determining the characteristic qualities of bacterial smear surface-ripened cheeses, the role of the

extracellular enzymes of *Arthrobacter* spp. has probably been underestimated with respect to other surface bacteria species such as *B. linens*. Apart from a few studies on the enzymology of soil *Arthrobacter* spp. [3, 24, 33], to our knowledge only proteolytic enzymes (2 extracellular proteinases and an extracellular proline iminopeptidase) from dairy *A. nicotianae* have been purified and characterized [45, 46].

In this article, we report on the purification and characterization of an extracellular esterase from *A. nicotianae* 9458, a typical species isolated from the bacterial smear of surface-ripened cheeses.

2. MATERIALS AND METHODS

2.1. Microorganism, growth conditions and harvest

Arthrobacter nicotianae 9458 was obtained from the culture collection of the University College Cork, Ireland. *A. nicotianae* 9458 was precultivated and cultivated (7 L) in nutrient broth, supplemented with yeast extract (0.5%, w/v), at 28 °C under shaking conditions (150 rpm) for 72 h. The cells were removed by centrifugation at 16 000 g for 15 min at 4 °C in order to obtain the cell-free supernatant. Cells were in the late exponential growth phase. Microscopic observations showed short- to medium-length rod-shaped cells; coccoid cells, which appear in stationary phase culture, were not present. Moreover, intracellular aminopeptidase activity, assayed as described by El-Soda and Desmazeaud [15] using leucine- p-nitroanilide (2 mmol·L^{-1} final concentration) in methanol as substrate, was not detected in the cell-free supernatant. The cell-free supernatant was freeze-dried.

2.2. Chemicals

Prepacked fast protein liquid chromatography (FPLC) columns of Phenyl-Superose HR5/5, Mono-Q HR5/5 and Superose 12 HR10/30 were obtained from Pharmacia-Biotech, Uppsala, Sweden. DEAE-cellulose, Sepharose 6B, inhibitors, substrates, Fast Garnet GBC sulfate salt, Coomassie Brilliant Blue R250 and protein molecular weight standards were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.3. Enzyme purification

The freeze-dried supernatant preparation from the cultivation of *A. nicotianae* was resuspended in 50 mmol·L^{-1} potassium phosphate (KPi) buffer, pH 7.0, and dialyzed for 24 h at 5 °C against the same buffer.

The sample was applied to a DEAE-cellulose anion exchange column (50×1.6 cm inside diameter) and proteins were eluted at a flow rate of 54 mL·h^{-1} with a linear NaCl gradient (0 to 0.5 mol·L^{-1}) in 50 mmol·L^{-1} KPi buffer, pH 7.0 (gradient volume, 500 mL). Active fractions were pooled, dialyzed against 50 mmol·L^{-1} KPi buffer, pH 7.0, concentrated 10-fold by freeze-drying and subjected to gel filtration on Sepharose 6B (70×2.0 cm inside diameter). The elution was carried out with 450 mL of 50 mmol·L^{-1} KPi buffer, pH 7.0, containing NaCl 0.15 mol·L^{-1}, at a flow rate of 42 mL·h^{-1}. Active fractions were pooled, dialyzed against distilled water, freeze-dried, resuspended in 50 mmol·L^{-1} KPi buffer, pH 7.0, containing 1.7 mol·L^{-1} (NH$_4$)$_2$SO$_4$ and applied to a Phenyl-Superose HR5/5 hydrophobic interaction column. After loading the sample, the protein was eluted at a flow rate of 24 mL·h^{-1} with a linear (NH$_4$)$_2$SO$_4$ gradient from 1.7 to 0 mol·L^{-1} in 50 mmol·L^{-1} KPi buffer, pH 7.0 (gradient volume, 25 mL). Active fractions were pooled, dialyzed, freeze-dried and resuspended in 20 mmol·L^{-1} KPi buffer, pH 7.0. Esterase was then purified on an FPLC Mono-Q HR5/5 column by eluting with a linear NaCl gradient from 0 to 0.5 mol·L^{-1} at a flow rate of 45 mL·h^{-1} (gradient volume, 20 mL).

2.4. Protein determination

During the purification steps, the protein was determined by using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as standard. The elution profile of the protein for conventional column chromatography was monitored by measuring absorbance at 280 nm.

2.5. Enzyme assays

Esterase activity (acetyl ester hydrolase, EC 3.1.1.6) during purification was determined as described by Gobbetti et al. [23], using β-naphthyl butyrate. The assay mixture contained 160 μL of 50 mmol·L^{-1} KPi buffer, pH 7.0, 20 μL of β-naphthyl butyrate (5 mmol·L^{-1} in methanol; final concentration 0.5 mmol·L^{-1}) and 20 μL of enzyme solution. After incubation for 2 h at 30 °C, the colour was developed by adding 0.6 mL of Fast Garnet GBC sulfate salt preparation (0.25 mg·mL^{-1} in 10% (w/v) sodium dodecyl sulphate) followed by incubation at room temperature for 15 min. Absorbance was measured at 560 nm and, using a standard curve, the specific activity was expressed as μmol of β-naphthol released per min per mg of protein at 30 °C and pH 7.0. Other β-naphthyl esters of C2 to C18:1 fatty acids were used to determine substrate specificity. To determine the relationships between hydrolytic velocity and substrate concentration, the enzyme solution was incubated at 30 °C with various concentrations of β-naphthyl butyrate ranging from 0.3 to 1.8 mmol·L^{-1}. The activity was maximum at a substrate concentration of 0.5 mmol·L^{-1} (which was the final substrate concentration used in the assays). The Lineweaver-Burk plot showed that the reaction followed a Michaelis-Menten kinetic.

Lipase activity (acylglycerol acylhydrolase, EC 3.1.1.3) was measured by the release of free fatty acids (FFA) from various triglycerides (from C4 to C18:1) and from milk fat, as described by Dupuis et al. [9]. The emulsion was prepared by sonicating at maximum amplitude for 3 min (Sony Prep, model 150, Sanyo, UK) a 10% (w/v) aqueous solution of gum arabic and 10% (v/v) triglyceride mixture. After cooling in an ice-water-ethanol bath, 200 μL of enzyme solution were added to 500 μL of emulsion (final concentration of triglycerides, 0.11 mol·L^{-1}), 800 μL of 50 mmol·L^{-1} KPi, pH 7.0, containing sodium azide (final concentration, 0.02%). After incubating for 12 h at 30 °C, FFA were extracted from the acidified (at pH 3.0 with 1 mol·L^{-1} HCl) reaction mixture by a 60:40 mixture of diethyl ether/light petroleum and titrated with 0.05 mol·L^{-1} alcoholic KOH to pH 10.0 using an auto-titrator [19]. A unit of activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of FFA per min.

2.6. Enzyme characterization

2.6.1. Molecular mass measurement

The relative molecular mass of the purified esterase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by FPLC gel filtration. SDS-PAGE was conducted according to the Laemmli procedure [28]; the gels contained 10% acrylamide (separation distance, 10 cm; gel thickness, 1 mm) and were stained with Coomassie Brilliant Blue R250. Bovine albumin (66 kg·mol^{-1}), egg albumin (45 kg·mol^{-1}), pepsin (34.7 kg·mol^{-1}) and trypsinogen (24 kg·mol^{-1}) were used as molecular mass standards. Gel filtration was conducted on a Superose 12 HR 10/30 column calibrated with the above protein molecular weight standards. Equilibration of the column and elution were performed using 50 mmol·L^{-1} KPi buffer, pH 7.0, containing 0.15 mol·L^{-1} NaCl. 100 μL of each standard protein or enzyme solution were applied to the column and eluted at a flow rate of 0.5 mL·min^{-1}.

2.6.2. pH and temperature optima

The enzyme activity in the pH range of 4-11 was determined at 30 °C, using a universal buffer composed of boric acid (0.57 mol·L^{-1}), citric acid (0.33 mol·L^{-1}), NaH$_2$PO$_4$ (0.33 mol·L^{-1}), NaOH (5 mol·L^{-1}), and various amounts of 1 mol·L^{-1} HCl [7]. The enzyme activity was also determined in 50 mmol·L^{-1} KPi buffer, pH 7.0, over a temperature range of 5-60 °C. In both cases β-naphthyl butyrate was used as substrate.

2.6.3. Heat stability

Portions (40 μL) of the purified enzyme in 50 mmol·L^{-1} KPi, pH 7.0, were heated in capillary glass tubes at 40-70 °C for several min. At intervals, samples were cooled and the residual activity was measured at 30 °C on β-naphthyl butyrate, as described previously.

2.6.4. Effect of inhibitors, divalent cations and NaCl

The effect of inhibitors or divalent cations was determined after pre-incubating the enzyme solution with chemical reagents (final concentration 4 mmol·L^{-1}), for 20 min at 30 °C in 50 mmol·L^{-1} KPi, pH 7.0. The reaction was initiated by adding β-naphthyl butyrate and the activity was measured after incubating at 30 °C for 2 h.

To determine the sensitivity to NaCl, the enzyme solution was incubated for 2 h at 30 °C in 50 mmol·L^{-1} KPi, pH 7.0 in the presence of β-naphthyl butyrate and 0, 1, 2, 5 or 10% NaCl.

3. RESULTS

3.1. Enzyme purification

Results from purification of the extracellular esterase from *A. nicotianae* 9458 are summarized in Table I. Chromatography on both DEAE-cellulose and on Sepharose 6B resolved the esterase activity into one peak corresponding to the major protein peak (data not shown). Further purification was achieved by FPLC on a Phenyl-Superose column; the esterase was eluted at 0.0 mol·L^{-1} (NH$_4$)$_2$SO$_4$. The

Table I. Purification of an extracellular esterase from *Arthrobacter nicotianae* 9458.

Tableau I. Purification d'une estérase exocellulaire isolée d'*Arthrobacter nicotianae* 9458.

Purification step	Total protein (mg)	Total activity[a] (μmol·min^{-1})	Specific activity[a] (μmol·mg^{-1}·min^{-1})	Purification factor (fold)	Activity yield (%)
Dialyzed retentate of cell-free supernatant	135.4	980.4	7.2	1.0	100
DEAE-cellulose	20.6	569.8	27.7	3.8	58
Sepharose 6B	7.2	455.3	63.2	8.7	46
Phenyl-Superose	1.6	258.9	161.8	22.5	26
Mono-Q	0.7	171.1	244.4	33.9	17

[a] Esterase activity during purification was determined as described in Section 2.4, using β-naphthyl butyrate as substrate. After incubation for 2 h at 30 °C, the colour was developed by adding 0.6 mL Fast Garnet GBC sulfate salt. The specific activity was expressed as μmol of β-naphthol released per min per mg of protein at 30 °C and pH 7.0.

[a] L'activité de l'estérase au cours de la purification était effectuée comme décrit en section 2.4, en utilisant le β-naphthyl butyrate comme substrat. Après incubation 2 h à 30 °C, la couleur était développée par ajout de 0,6 mL de sulfate Fast Garnet GBC. L'activité spécifique était exprimée en μmol de β-naphthol libéré par min et par mg de protéine à 30 °C et à pH 7,0.

enzyme was then purified by chromatography on a Mono-Q anion exchange column; purified esterase was eluted at 0.33 mol·L^{-1} NaCl. After the final anion exchange chromatography, the enzyme was homogeneous by SDS-PAGE with a 34-fold purification, a final recovery of 17% and a specific activity of 244 µmol·min^{-1}·mg^{-1}. This enzyme preparation was used for the subsequent biochemical characterization.

3.2. Enzyme characterization

3.2.1. Molecular mass measurement

The purified enzyme showed a single band on SDS-PAGE, corresponding to a molecular mass of about 32 kg·mol^{-1} (Fig. 1). Approximately the same value was estimated by FPLC gel filtration on Superose 12 HR10/30 (data not shown), suggesting that the enzyme is a monomer.
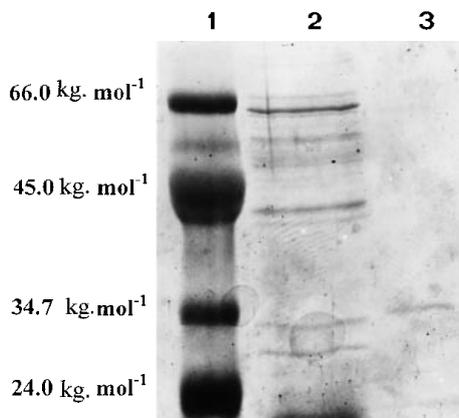

Figure 1. SDS-PAGE of the extracellular esterase from *Arthrobacter nicotianae* 9458. Lanes: 1, reference proteins (see Materials and Methods); 2, cell-free supernatant; 3, purified preparation.
Figure 1. SDS-PAGE de l'estérase exocellulaire purifiée d'*Arthrobacter nicotianae* 9458. Lignes : 1, protéines de référence (voir Materials and Methods) ; 2, surnageant ; 3, estérase exocellulaire.

3.2.2. pH and temperature optima and heat stability

The optimum pH for the esterase was 7.0 and it retained 50, 75 and 90% of its maximum activity at pH 5.5, 6.0 and 6.5, respectively (Fig. 2). At pH 7.0, the optimum temperature was 30 °C; about 37 and 55% of the maximum activity was expressed at 10 and 15 °C, respectively (Fig. 2). The activity rapidly decreased above 40 °C.

The enzyme retained 60% of the maximum activity after heating for 10 min at 45 °C and was completely inactivated by heating for 1 min at 65 °C.

3.2.3. Substrate specificity

The highest activity on β-naphthyl esters was that determined on β-naphthyl butyrate (Tab. II). The activity ranged from 36 to 74% of the maximum when β-naphthyl esters of C2 to C10 fatty acids were used as substrates. No activity was detected on β-naphthyl esters of C12 to C18:1 fatty acids.

Among the triglycerides, the maximum activity was detected on tributyrin, and the activity on tricaproin was 22% of that found on tributyrin. Triglycerides of C8 to C18:1 and milk fat were not hydrolyzed (Tab. II).

3.2.4. Effect of inhibitors, divalent cations and NaCl

Extracellular esterase from *A. nicotianae* 9458 was markedly inhibited by the serine enzyme inhibitor PMSF, and to a lesser extent (42% of the maximum) by the metal chelator, EDTA (Tab. III). The reducing agent dithiothreitol (DTT) and the sulphydryl-reactive reagent iodoacetic acid showed a low inhibitory effect. The thiol-blocking agent *p*-HMB had no effect. The enzyme was moderately stimulated by the sulphydryl-reactive reagent *N*-ethylmaleimide (NEM). Several divalent cations, i.e., Ca^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} partially inhibited the enzyme, which was completely
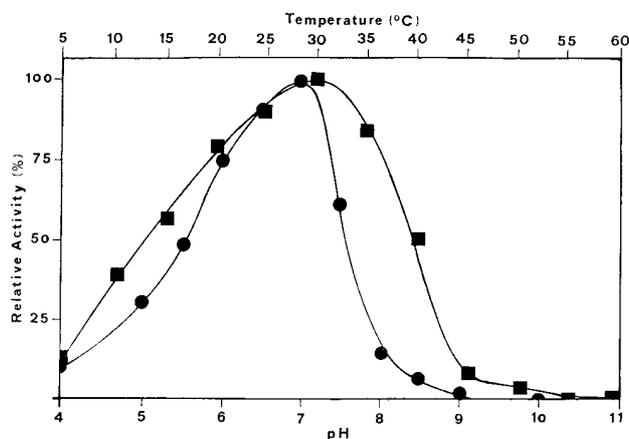
Figure 2. Effect of pH (●) and temperature (■) on the activity of the purified extracellular esterase from *Arthrobacter nicotianae* 9458. Esterase activity was determined as described in Section 2.6.2., using β-naphthyl butyrate as substrate. After incubation for 2 h at different pH or temperatures, the colour was developed by adding 0.6 mL Fast Garnet GBC sulfate salt.

Figure 2. Effect du pH (●) et de la température (■) sur l'activité de l'estérase exocellulaire purifiée, produite par *Arthrobacter nicotianae* 9458. L'activité de l'estérase a été déterminée en présence du substrat β-naphthyl butyrate, comme décrit dans la section 2.6.2. Après une incubation de 2 h à différentes valeurs de pH ou température, la couleur a été développée après l'addition de 0,6 mL de sulfate Fast Garnet GBC.

Table II. Relative activity (%) of the extracellular esterase from *Arthrobacter nicotianae* 9458 on β-naphthyl esters, triglycerides of fatty acids and milk fat.

Tableau II. Activité relative (%) de l'estérase exocellulaire isolée d'*Arthrobacter nicotianae* 9458 mesurée sur les esters β-naphthyl, les triglycérides des acides gras et sur la matière grasse du lait.

Substrate	Relative activity[a] (%)	Substrate	Relative activity[a] (%)
β-Naphthyl		**Triglycerides and milk fat**	
Acetate – C2	69	Tributyrin	100
Butyrate – C4	100	Tricaproin	22
Caproate – C6	74	Tricaprylin	0
Caprylate – C8	53	Tricaprin	0
Caprate – C10	36	Trilaurin	0
Laurate – C12	0	Trimiristin	0
Myristate – C14	0	Tripalmitin	0
Palmitate – C16	0	Tristearin	0
Stearate – C18	0	Milk fat	0
Oleate – C18:1	0		

[a] The relative activity on β-naphthyl esters in solution and on triglycerides of fatty acids in emulsion was calculated assuming the activity of 10 units of esterase on β-naphthyl butyrate and tributyrin to be 100%.

[a] L'activité relative sur les esters de β-naphthyl en solution et sur le triglycérides d'acides gras en émulsion était calculée en attribuant la valeur de 100 % à l'activité de 10 unités d'estérase sur le butyrate de β-naphthyl et la tributyrine.

inactivated by Fe^{2+} and Sn^{2+}. Mn^{2+} showed a low inhibitory effect, while Mg^{2+} had no effect on activity.

The activity of the extracellular esterase was not inhibited at pH 7.0 and 30 °C by concentrations of NaCl up to 5%, but was moderately reduced in the presence of 10% NaCl (data not shown).

4. DISCUSSION

A 32 kg·mol^{-1} extracellular esterase from *A. nicotianae* 9458 was purified to homogeneity and characterized.

Table III. Effect of chemical reagents and divalent cations on the activity of the extracellular esterase from *Arthrobacter nicotianae* 9458.

Tableau III. Effet des réactifs chimiques et des cations divalents sur l'activité de l'estérase exocellulaire d'*Arthrobacter nicotianae* 9458.

Reagent[a] or cation	Residual activity[b] (%)
EDTA	42
PMSF	15
NEM	116
Iodoacetic acid	78
DTT	75
p-HMB	88
Ca^{2+}	14
Fe^{2+}	0
Cu^{2+}	29
Zn^{2+}	30
Hg^{2+}	22
Mn^{2+}	63
Mg^{2+}	96
Ni^{2+}	29
Sn^{2+}	0

[a] EDTA: ethylenediaminetetracetic acid; PMSF: phenylmethylsulfonyl fluoride; NEM: *N*-ethylmaleimide; DTT: dithiothreitol; *p*-HMB: *p*-hydroxy mercuric benzoate.
[b] 100% activity represents the enzyme activity toward β-naphthyl butyrate with no reagent or cation added.
[b] Une activité de 100 % représente l'activité de l'enzyme sur le butyrate de β-naphthyl sans ajout de réactif ou de cation.

At the end of ripening, the surface microflora of bacterial smear surface-ripened cheeses is dominated by acid-sensitive bacteria, such as *Brevibacterium*, *Micrococcus*, *Corynebacterium* and *Arthrobacter* [12]. Mixed cultures, suitable for surface ripening, have been developed, and include *Arthrobacter* spp. [11].

Although *Arthrobacter* spp. together with *B. linens* are the main microorganisms found on several bacterial smear surface-ripened cheeses, until this study was carried out the esterases and lipases of dairy *Arthrobacter* spp. had not been studied.

The extracellular esterase from *A. nicotianae* 9458 is a monomer with a molecular mass of about 32 kg·mol^{-1}. While the intracellular esterase from *B. linens* ATCC 9174 is a tetramer with a molecular mass of 201 kg·mol^{-1} and is composed of four 54 kg·mol^{-1} subunits [43], 3 esterases isolated from *Brevibacterium* sp. R312 were monomers with molecular masses of 38, 45 and 56 kg·mol^{-1} [29]. Esterases purified from *Lactococcus lactis* ssp. *lactis* [48], *Lactobacillus plantarum* [21] and *Lc. fermentum* [23] are monomeric, with molecular masses of 64, 85 and 67 kg·mol^{-1}, respectively.

The purified esterase from *A. nicotianae* 9458 is inhibited by several cations (Ca^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+} and Zn^{2+}) and is completely inactivated by Fe^{2+} and Sn^{2+}; Mg^{2+} had no effect on activity. The intracellular esterase from *B. linens* ATCC 9174 was strongly inhibited by Cu^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+} and Hg^{2+}, while Mg^{2+} and Ca^{2+} had no effect on activity [43]. Three partially purified esterases from *Brevibacterium* sp. R312 were inhibited by Hg^{2+} and were not activated by Mg^{2+} or Ca^{2+} [29]. Purified esterases from *Micrococcus* spp. [2] and from *Lc. lactis* ssp. *lactis* [48] were inhibited by Hg^{2+} and Cu^{2+}; Mg^{2+} and Ca^{2+} showed no effect on enzyme activity. However, it has been reported that Mg^{2+} and Ca^{2+} increased the activity of the esterases purified from *L. plantarum* [21] and *L. fermentum*

[23] which, similarly to the extracellular esterase from *A. nicotianae* 9458, were inhibited by Hg^{2+}. The extracellular esterase from *A. nicotianae* 9458 is a serine enzyme that is inhibited by PMSF and stimulated by NEM. Similarly to the serine esterase from *L. fermentum* [23], the activity of the enzyme of *A. nicotianae* 9458 was affected by EDTA and iodoacetic acid but not by *p*-HMB.

Esterases hydrolyse esters in solution, and may also hydrolyze tri- and especially di- and monoglycerides containing short-chain fatty acids. Lipases hydrolyze esters in emulsion and occasionally water-soluble substrates [6, 27]. The enzyme examined in this study is an esterase because it strongly hydrolyzed water-soluble β-naphthyl esters of fatty acids from C2 to C8 (hydrolysis of C10 also occurred) and, among the triglycerides, it hydrolyzed tributyrin, and to a lesser extent tricaproin in emulsion. The esterase from *A. nicotianae* 9458 clearly demonstrated a preference for short-chain fatty acid esters of β-naphthol. Compared to the activity on β-naphthyl butyrate, the activity on β-naphthyl acetate, caproate, caprylate and caprate was 69, 74, 53 and 36%, respectively (Tab. III). Esterases from *B. linens* [43, 47], *L. fermentum* [23], *L. plantarum* [21] and *Propionibacterium* spp. [8, 36] also showed a preference for short-chain fatty acid esters. Esterase from *L. fermentum* only hydrolyzed tributyrin among some triglycerides [23]. Woo et al. [50] quantified the major free fatty acids in several smear surface-ripened cheeses. Despite the variation in the concentrations and proportions of free fatty acids, such cheeses contained high levels of C4 and C6 free fatty acids which contribute to the cheese flavour. Compared to the blue-veined cheeses, the concentration of longer fatty acids is very low in smear surface-ripened cheeses. This characteristic indicates that the extracellular esterase from *A. nicotianae* 9458 may release C4 and C6 fatty acids during ripening and may contribute to flavour development.

The extracellular esterase of *A. nicotianae* 9458 had pH and temperature optima of 7.0 and 30 °C, respectively. Similarly, crude preparations of esterases from lactic acid bacteria [16, 26, 27] and purified esterases from *L. casei* ssp. *casei* [31], *L. plantarum* [21, 38] and *L. fermentum* [23] had pH and temperature optima at 6.5–7.0 and 30–40 °C, respectively. The intracellular as well as the cell-bound esterase from *B. linens* showed pH and temperature optima of about 7.5 and 35 °C, respectively [43, 47]. The extracellular esterase of *A. nicotianae* 9458 retained 90 and 55% of the maximum activity at pH 6.5 and 15 °C, respectively. In the case of bacterial smear surface-ripened cheeses, yeasts and moulds initially dominate the surface because they are acidotolerant and halotolerant. Growth of yeasts and moulds on the surface of the cheese results in an increase of pH, due to the combination of lactate utilization and ammonia production, which encourages the growth of less acid-tolerant microorganisms such as *Brevibacterium* spp., *Micrococcus* spp. and *Arthrobacter* spp. During ripening, the pH on the surface of Taleggio cheese increases from 5.2 to 6.5 [22].

Tolerance to or activation by NaCl is a prerequisite for the activity of bacterial enzyme in the smear of the surface-ripened cheeses. Similarly to extracellular proteinases [45] and proline iminopeptidase [46] from *A. nicotianae* 9458, the extracellular esterase from *A. nicotianae* 9458 is quite insensitive to NaCl.

Since the esterase of *A. nicotianae* 9458 is an extracellular enzyme which retains considerable activity at the pH, temperature and NaCl concentration of ripening and mainly releases short chain fatty acids, it may contribute greatly to flavour development in smear surface-ripened cheeses.

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