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Original article

Cloning and characterization of an esterase from *Propionibacterium freudenreichii* ssp. *shermanii*

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Abstract — An esterase gene, *est*A, from *Propionibacterium freudenreichii* ssp. *shermanii* was cloned in *Eschericia coli.* The clone, carrying a 3.1-kb insert, caused lipolysis on tributyrin agar during incubation at 4 °C. The insert was sequenced and analyzed. It revealed two open reading frames, ORF1 and ORF2. ORF1 has a capability to code for a protein of 41.8 kg·mol^{-1} (388 aa) and shares up to 38% identity with esterases or lipases from several bacteria. ORF2 encodes for a protein of 42.7 kg·mol^{-1} (379 aa) and shares 62% identity with CaiA, a carnitine operon oxidoreductase with a CoA-binding site. The lipolytic enzyme coded by ORF1 degraded *p*-nitrophenyl butyrate, *p*-nitrophenyl caproate and tributyrin, but not long chain fatty acid substrates like *p*-nitrophenyl palmitate or triolein triglyceride. Based on the preference for short chain fatty acids, and the homology profiles, the ORF1 was named an esterase, EstA. The esterase degraded *p*-nitrophenyl butyrate between 4 °C and 55 °C, the optimum being at 37 °C. The highest activity was detected at pH 8.

esterase / cloning / *Propionibacterium*

Résumé — **Clonage et caractérisation d'une estérase produite par *Propionibacterium freudenreichii* ssp. *shermanii.*** Un gène d'estérase, *est*A, de *Propionibacterium freudenreichii* ssp. *shermanii* a été cloné dans *E. coli*. Le clone portant un insert de 3,1 kb a causé, après incubation à 4 °C, la lipolyse d'un substrat tributyrine inclus dans la gélose. L'insert a été séquencé et analysé. Il a révélé deux cadres ouverts de lecture, ORF1 et ORF2. ORF1 a la capacité de coder une protéine de 41,8 kg·mol^{-1} (388 aa) et présente jusqu'à 38 % d'identité avec les gènes d'estérases ou de lipases de plusieurs bactéries. ORF2 code pour une protéine de 42,7 kg·mol^{-1} (379 aa) et présente 62 % d'identité avec CaiA, une carnitine oxydoréductase possédant un site de liaison CoA. ORF1 hydrolyse le butyrate de *p*-nitrophényle, le caproate de *p*-nitrophényle et la tributyrine, mais pas le palmitate de *p*-nitrophényle et la trioléine contenant des acides gras à chaîne longue. Du fait de sa préférence pour les

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acides gras à chaîne courte et de son profil d'homologie, ORF1 a été appelé estérase EstA. Cette estérase dégradait le butyrate de *p*-nitrophényle entre 4 °C et 55 °C, l'optimum étant à 37 °C. L'activité la plus élevée était détectée à pH 8.

estérase / clonage / *Propionibacterium*

1. INTRODUCTION

Propionibacterium freudenreichii plays an important role in Swiss-type cheese ripening and flavor production by producing propionic acid, acetic acid and CO_2. Also proline iminopeptidase, degrading proline-rich peptides of milk proteins, is assumed to have a positive effect on the flavor. Because of its great effect on the structure and taste of dairy products, proteolytic activity has been intensively studied. In contrast lipolysis, comprising lipase and esterase enzymes, seems to need more comprehensive studies. Lipolytic activity from psychrotrophic microorganisms is generally thought to impair the quality of milk and cheese [5], though, the lipolytic activity of starters may also produce tasty aromatic compounds during cheese ripening [27, 28].

Esterases have been purified and characterized from several dairy lactic acid bacteria [4, 12, 13, 15, 16]. Lipase and esterase activities of dairy *Propionibacterium freudenreichii* have also been studied [9, 11]. These reports showed that the intracellular fraction of propionibacterium contained between three to six different esterase activities. An extracellular lipase activity was also detected.

Only one lipase gene of propionibacteria, *geh*A, from a clinical isolate of *Propionibacterium acnes* has been studied in detail [24]. This gene coded for an extracellular enzyme with a molecular mass of 36 kg·mol^{-1}. An active lipase (33 kg·mol^{-1}) was produced after the cleavage of a signal sequence of 26 amino acids. Though esterase genes have been cloned from several other microorganisms [8], this is the first report of a gene from dairy propionibacteria coding for a fatty acid-ester degrading enzyme.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table I. *Propionibacterium freudenreichii* was grown anaerobically at 30 °C on a YEL-agar (yeast extract-lactate) [20] or in a YEL medium. *E. coli* was grown in Luria-Bertani (LB) broth or on LB-agar at 37 °C [2]. Lipolytic activity was tested on tributyrin (1% v/v) and triolein plates (0.1% v/v) [1]. Where appropriate, ampicillin (Sigma Chemical Co., St. Louis, MO, USA) 100 μg·mL^{-1}, X-gal (Sigma) 40 μg·mL^{-1} and IPTG (isopropyl-B-D-thiogalactopyranoside, Sigma) 0.5 mmol·L^{-1} were added.

2.2. Plasmid and genomic DNA isolations

Glycine (0.5%) was added into the growth medium of *P. freudenreichii* and the cells were grown from 5 h to overnight. Chromosomal DNA was isolated as described earlier [30]. Plasmid DNA isolation from *E. coli* was performed with a Wizard plasmid isolation kit (Promega, Madison, Wisconsin, USA).

2.3. Construction and screening of a plasmid library

The genomic DNA of *Propionibacterium* was partially digested with *Sau*3AI

restriction enzyme and fragments of 3–5 kb were purified from a SeaPlaque agarose gel (FMC BioProducts, Rockland, ME, USA). Fragments were ligated to the *Bam*HI restriction site of a calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA, USA) treated pUC18 vector. The ligation mix was electroporated into *E. coli* [2] and the cells were plated on tributyrin agar. The plates were incubated at 37 °C overnight and then moved to 4 °C for at least for 7 d. Lipolytic clones producing a clear halo around the colony were picked for further studies. Subclones of the 3.1-kb insert were made in pUC18 by exploiting the *Pst*I, *Hind*II and *Eco*RI sites in the insert (Tab. I). The ligated fragments were electroporated into *E. coli* TG1 and screened with blue-white selection.

2.4. Nucleotide sequencing and analysis

Both strands of the insert were sequenced with pUC18 specific primers or sequence specific oligonucleotides. The automatic sequencing (ALF) service from the Institute of Biotechnology, University of Helsinki, Finland was used. The sequence was reconstructed using the WWW-server BCM (Baylor College of Medicine) Search Launcher: Pairwise Sequence Alignment and Sequence Utilities. The sequence was analyzed in 6 different reading frames using BLAST BlastX - program (NCBI) against the Genbank database (release March 30, 2001). Alignments were performed with CLUSTALW and BLAST CD-search. The GenBank accession number of the sequence is AF159497.

2.5. Hybridization

For hybridization the *est*A gene fragment of 800 bp and the acyl-CoA dehydrogenase fragment of 450 bp were isolated from plasmid pVS120 by exploiting the *Nco*I and *Sal*I restriction sites of the insert, respectively. The probes were labeled by random priming with the DIG DNA Labeling and Detection Kit (Boehringer Mannheim). Southern hybridization was performed according to the instructions of the Boehringer Mannheim

Table I. Bacterial strains and plasmids used in this study.

Plasmid or strain	Description	Reference or source
Propionibacterium freudenreichii ssp. *shermanii* JS		Valio Ltd, Finland
Escherichia coli TG1		[14]
pUC18	cloning vector	[26]
pVS120	pUC18 derivative containing a 3.1- kb insert of *P. freudereichii* ssp. *shermanii* JS genomic DNA	this work
pVS122	pUC18 derivative containing a 2-kb *Sac*I-*Hin*dII fragment of pVS120	this work
pVS123	pUC18 derivative containing a 1-kb *Sac*I-*Pst*I fragment of pVS120	this work
pVS125	pUC18 derivative containing a 2-kb *Eco*RI-*Xba*I fragment of pVS120	this work

DIG system at 68 °C for 20 h. Washings were at room temperature with 2 × SSC - 0.1% SDS (20 × SSC; 3 mol·L^{-1} NaCl - 0.3 mol·L^{-1} trisodiumcitrate, pH 7.0) for 2 × 5 min, and at 68 °C with 0.1 × SSC - 0.1% SDS for 2 × 15 min. Colorimetric detection was performed according to the instructions in the DIG DNA Labeling and Detection Kit (Boehringer Mannheim).

2.6. Degradation of fatty acid-esters

E. coli cells were collected (5 000 *g*, 10 min) from 30 mL of log-phase culture and were washed with 5 mL of 50 mmol·L^{-1} Tris-HCl (pH 8). The cells were resuspended in 2 mL of buffer and were sonicated on a water-ice bath for 1 min in 700 µL aliquots. After centrifugation (9 300 *g*, 5 min, 4 °C) the supernatant was frozen in aliquots. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

The effect of temperature on the enzyme activity was studied by preparing a mixture containing 100 µL of enzyme preparation, 25 µL *p*-nitrophenyl butyrate (9 mmol·L^{-1} in isopropanol), and 975 µL of buffer (50 mmol·L^{-1} Na_2HPO_4 - 5.75 mmol·L^{-1} Na-deoxycholate - 0.11% gum arabic, pH 8) [23]. The mixture was incubated for 20 min at 4 °C, 20 °C, 37 °C, or 55 °C after which the absorbance was measured at 400 nm against the buffer-substrate mixture (autohydrolysis background), which had been incubated in similar conditions. The host organism of the clones, *E. coli* TG1 containing vector pUC18, was analyzed beside the sample and the absorbance of the host organism was subtracted from that of the sample. The relative activity of the sample at different temperatures was calculated by defining the highest absorbance value as 100%.

In subsequent studies the autohydrolysis of the substrate and the activity of the *E. coli* host organism were analyzed and subtracted from the sample values as described above. The effect of pH (pH 4–11) was studied in a buffer without gum arabic and Na-deoxycholate because of precipitation of those substances at low pH. The assays at pH 9 and 10 were performed using boric acid (57 mmol·L^{-1}) - citric acid (33 mmol·L^{-1}) - NaH_2PO_4 buffer (33 mmol·L^{-1}) [4]. All the assays were done at 20 °C using 60-min incubation time.

The substrate specificity of the enzyme was determined in a buffer of 50 mmol·L^{-1} Na_2HPO_4 - 5.75 mmol·L^{-1} Na-deoxycholate - 0.11% gum arabic, pH 8, using *p*-nitrophenyl derivative of butyrate (C4), caproate (C6), caprylate (C8), caprate (C10) or palmitate (C16) as a substrate.

3. RESULTS AND DISCUSSION

3.1. Cloning of lipolytic activity

Several *Propionibacterium* sp. strains were screened for lipolysis on tributyrin agar plates. This medium is not specific for either lipase or esterase but shows the ability of the strain to degrade a short-chain triglyceride. Most strains were only weakly lipolytic and some possessed no lipolytic activity at all. *P. freudenreichii* ssp. *shermanii* JS was among the most lipolytic strains, producing a clear halo around the colonies (data not shown). However, as a whole the lipolytic activity of the dairy propionibacteria studied seemed to be quite weak.

A pUC18-based plasmid library, containing *P. freudenreichii* ssp. *shermanii* JS genomic DNA inserts, was constructed in *E. coli* TG1 and screened for lipolytic activity. More than 10 000 *E. coli* colonies were screened. Only four of the clones produced a clear halo on tributyrin agar during incubation at 4 °C. Plasmid DNA was isolated from the lipolytic clones. A plasmid containing a 3.1-kb insert was designated pVS120 and was further analyzed.

3.2. DNA sequence and protein homology analysis

Sequencing of the 3.1-kb insert revealed two open reading frames, ORF1 and ORF2. ORF1 consists of 388 and ORF2 of 379 amino acids and they have a coding capacity for 41.8 and 42.7 kg·mol^{-1} proteins, respectively. The G + C content of ORF1 and ORF2 (63.2–68.0%) and the average G + C content for the whole *P. freudenreichii* genomic insert (3160 nt; GC% 65.75%) fits the G + C percent of *P. freudenreichii* (62–67%) well [6]. There is a strong bias in codon usage because 50% of the ORF1 codons had C as the third nucleotide. The same is reported in the pyrophosphate-dependent phosphofructokinase gene of *P. freudenreichii* [19].

A ribosome binding site (AGAAG) was found 4 nucleotides before the ATG start site of ORF1 and six nucleotides upstream of ORF2 (AGGGA). A putative promoter area starting at position 96 nt and ending at 145 nt was recognized in front of ORF1 by NNPP procaryote promoter predictor (Berkeley Drosophila genome project). Also a stem-loop structure (1362–1391 nt) between ORF1 and ORF2 was found. The promoter areas of *Propionibacterium* have not been analyzed extensively, though putative promoter areas have been suggested and several upstream sequences of ORFs have been published [17, 19, 21, 25, 29].

The deduced amino acid sequence of ORF1 is homologous to several esterases and lipases. The highest identity (40–38%) and similarity (54–51%) was found in *E. coli* acetyl esterase and lipase. Because of the homology with esterases and the specificity for short chain fatty acid substrates (see Sect. 3.3.), ORF1 was designated an esterase, EstA. Lipases and esterases generally contain Ser-His-(Asp/Glu) residues at the active site [7]. Serine is in many cases conserved within a Gly-X-Ser-X-Gly (GXSXG) motif where the beta-strand and alpha-helix turns [3]. Drablos and Petersen [8] have studied the consensus sequences for the whole family of esterases and lipases and found that also Gly-122, Gly-123 and Gly-124 are conserved, as well as the active site residues Ser-209, Glu-341 and His-449. The EstA of *P. freudenreichii* has the GXSXG-motif, GDSGG, serine at the position 215 aa and the three glycines (GGG) at the positions 148–150 aa (Fig. 1).

```
Plip        56 AAAGELDARLYRPLEED---NLPLLVFFHGGGFVMGNLDTHDNLCRSLASQTEAVVVSVA
Drlip       66 GAEGSLPARLYHPAGQAPASGWPLTVFFHGGGFVVYDLDTHDALCRELCATSGAAVLSVA
Aaest       55 LPGRTLKVRXYRPEGVEP--PYPALVYYHGGGWVVGDLETHDPVCRVLAKDGRAVVFSVD
Afest       63 GRNGDIRVRVYQQKPDS----P-VLVYYHGGGFVICSIESHDALCRRIARLSNSTVVSVD
Pfest      120 TAYGAVSVRQYRPAGPAPQDTLPVIVFIHGGGWVVGNPTTHDRITRILADSTKACVVSVD
Ecaes       67 TKYGQVETRLFCPQ----PDSPATLFYLHGGGFILGNLDTHDRIMRLLASYSQCTVIGID
                     : .* :              .: ****::: .  :** : * :.    . *..:

Plip       113 YRLAPENHFPAAPLDCYAATCWLVEHAAELGVDGRRLALAGDSAGGNLALAVSRLAAQRQ
Drlip      126 YRLAPEARFPAPVDDALASVVWAAAHAAELGADAGRLAVAGDSAGANLATVTALRSRDEG
Aaest      113 YRLAPEHKFPAAVEDAYDALQWIAERAADFHLDPARIAVGGDSAGGNLAAVTSILAKERG
Aaest      118 YRLAPEHKFPAAVYDCYDATKWVAENAEELRIDPSKIFVGGDSAGGNLAAAVSIMARDSG
Pfest      180 YTLSPEAKYPQALNECVAVVRALVDAPDEWGIDADDISLAGDSGGASLAFGVALHLRDEE
Ecaes      123 YTLSPEARFPQAIEEIVAACCYFHQQAEDYQINMSRIGFAGDSAGAMLALASALWLRDKQ
                   * *:** ::* .  :           . :   :   : ..***.*. **   :    :
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Figure 1. CLUSTALW alignment of *P. freudenreichii* esterase with most similar lipases and esterases. Only part of the amino acid sequences are shown. Identical residues (*) and conserved strong (:) and weak (.) groups are marked. GXSXG-motif and a triple glycine sequence are shown in bold. Plip, *Pseudomonas* sp. lipase; Drlip, *Deinococcus radiodurans* lipase; Aaest, *Alicyclobacillus acidocaldarius* esterase; Afest, *Archaeoglobus fuldigus* esterase; Pfest, *P. freudenreichii* esterase; Ecaes, *E. coli* esterase.

The deduced amino acid sequence of ORF2 shares 61% identity with CaiA oxidoreductase [10] and isovaleryl-CoA dehydrogenase of *E. coli*, enzymes with a putative CoA-binding site, and 43–30% identity with several acyl-CoA dehydrogenases of both prokaryotic and eukaryotic origin. Moreover, three acyl-CoA dehydrogenase domains were found in ORF2 using the BLAST CD-search (Fig. 2). CaiA is needed in carnitine (3-hydroxy-4-trimethylaminobutyrate) metabolism in *E. coli* [10]. Mammalian acyl-CoA dehydrogenases are flavin adenine dinucleotide (FAD)-containing enzymes that catalyze the first step in the beta-oxidation of fatty acids [18, 22]. The role of the putative acyl-CoA dehydrogenase in the metabolism of *P. freudenreichii* remains to be solved.

The succession of ORF1 and ORF2 in *P. freudenreichii* ssp. *shermanii* JS genomic DNA was verified by hybridization using internal fragments of *estA* and putative acyl-CoA dehydrogenase genes as probes. Hybridization of both probes to a more than 3-kb *Pvu*I fragment and a 1.5-kb *Pst*I fragment was seen both in genomic DNA and in plasmid pVS120 (data not shown). Few examples of a similar kind of gene arrangement exist. *Streptomyces coelicolor* cosmid 8 also has a lipase gene, followed by a hypothetical protein of 142 amino acids and a putative oxidoreductase gene (accession AL035654). *Mycobacterium tuberculosis* H37Rv has a lipase/esterase and an oxidoreductase gene next to each other (accession Z83018).

3.3. Lipolytic activity

E. coli TG1 containing pVS120 or a deletion of it, pVS122, pVS123, or pVS125 or the vector pUC18, were grown on tributyrin agar plates to test their ability to hydrolyze this substrate. The lipolytic activity of the clones seen on a tributyrin plate (a clear halo) and the activity of the cell extracts in a spectrophotometric assay using chromogenic *p*-nitrophenyl butyrate as substrate are shown in Figure 3. Only the *estA* gene was needed for lipolytic activity. The cloning host *E. coli* TG1 (containing pUC18) showed weak lipolysis at 4 °C on tributyrin agar when incubated for more than four weeks. It also degraded *p*-nitrophenyl butyrate. However, the activity on *p*-nitrophenyl butyrate was always less than half of the activity detected in TG1 harboring the plasmid pVS120.

```
consensus     1 GKGFKYAMKELDMERLVIAAQALGIAQGALDEAIPYAKQRKQFGKPLAHFQLIQFKLADM 60
PfCoA       224 GNGFKRGVKDFDLERFEVAMTNYGTAYCAFEDAAKYANQRVQGGEAIARKQLIQLKFADM 283
1EGE_A      242 GAGFKVAMGAFDKTRPVVAAGAVGLAQRALDEATKYALERKTFGKLLVEHQAISFMLAEM 301
gi_1168281_ 229 GEGFKIAMKTLDGGRNGIAAQAVGIAQGALDAALQYAKERKQFGKSIAEQQGIAFKLADM 288
gi_1168282_ 227 GDGFHIAMANLNVGRIGIAAQALGIAEAALEHAVDYAKQRVQFGRPIAANQGISFKLADM 286

consensus    61 ATKLEAARLLLYRAAWLADRGRPTSKEAAMAKLFASEAAMQVADDAVQILGGVGYTNDYP 120
PfCoA       284 KVDITNMRNMLYEIAWKADNDQLGRGDCSMAKYYCSHAAARVVDNALQTLAGVGVTGEHR 343
1EGE_A      302 AMKVELARMSYQRAAWEVDSGRRNTYYASIAKAFAGDIANQLATDAVQILGGNGFNTEYP 361
gi_1168281_ 289 ATMIEASRLLTYQAAWLESSGLPYGKASAMSKLMAGDTAMKVTTEAVQIFGGYGYTKDYP 348
gi_1168282_ 287 ATRAEAARHLVYHAADLHNRGLNCGKEASMAKQFASDAAVKAALDAVQIYGGYGYMKDYP 346

consensus   121 VVERFYRDAKITQIYEGTSEIQRLVIARALL 150
PfCoA       344 VQRFYRDLRVDRVSGGTDEMMILAAGRgal 373
1EGE_A      362 VEKLMRDAKIYQIYEGTSQIQRLIVAREHI 391
gi_1168281_ 349 VERYMRDAKITQIYEGTQEIQRLVISRMLA 378
gi_1168282_ 347 VERLLRDAKVTQIYEGTNEIQRLIISKYLL 376
```

Figure 2. Alignment of the C-terminal domain of *P. freudenreichii* acyl-CoA dehydrogenase with the most similar C-terminal domains of different organisms. Identical amino acids are shown in grey. PfCoA, *P. freudenreichii*; 1EGEA, human; gi 1168281 and gi 1168282, *Bacillus subtilis*.

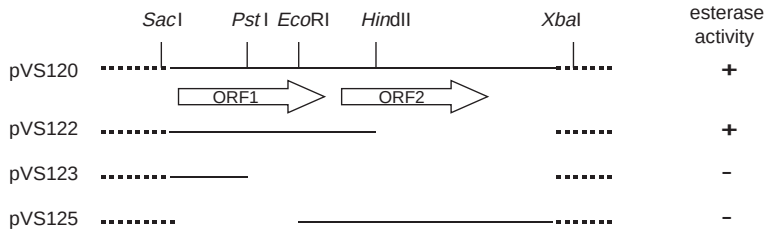

Figure 3. Schematic representation of the cloned chromosomal inserts of *P. freudenreichii* and the lipolytic activity of respective clones.

The clone containing pVS120 was as active towards *p*-nitrophenylcaproate (C6) as to *p*-nitrophenyl butyrate (C4), but showed only minimal activity towards *p*-nitrophenyl caprylate (C8) or caprate (C10). The cell extract of *E. coli* containing pVS120 was not able to degrade *p*-nitrophenyl palmitate (C16) faster than the control *E. coli* TG1. Moreover, neither TG1 (containing pUC18) nor the clone containing pVS120 produced a clear halo on triolein agar (data not shown).

The ability of the enzyme to degrade esters of butyrate (C4) and caprolate (C6) instead of a 16-carbon *p*-nitrophenyl palmitate indicates that the enzyme is an esterase rather than a lipase.

The esterase is not secreted into the culture medium, since neither the cell-free culture medium of *P. freudenreichii* ssp. *shermanii* JS nor the culture medium of *E. coli* TG1 containing pVS120 showed any esterase activity towards *p*-nitrophenyl butyrate. The fact that the formation of halos around colonies took several days instead of overnight may be due to the lack of excretion. Moreover, a signal sequence was not found in *estA*. In *P. freudenreichii* ssp. *shermanii* JS some activity towards *p*-nitrophenyl butyrate was detected with the sonicated cell debris. The activity was about 10% compared to the values of the cell extract.

Activity measurements using *p*-nitrophenyl butyrate as a substrate at 4 °C, 20 °C, 37 °C and 55 °C (pH 8) are shown in Figure 4. The highest activity was seen at 37 °C. At 20 °C and 4 °C the activity was 73 and 57% of the maximum, respectively. No activity was detected at 65 °C after 10 minutes' incubation (data not shown). Thus, the esterase has the capability to be active at the temperature of Swiss-type cheese ripening.

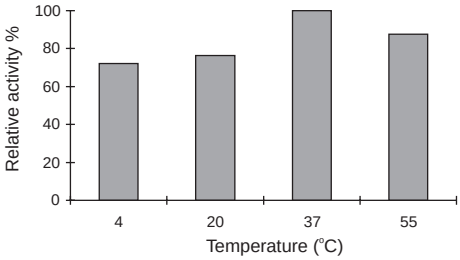

Figure 4. Esterolytic activity of cell extract of *E. coli* TG1 containing pVS120. Para-nitrophenyl butyrate is used as a substrate at 4 °C, 20 °C, 37 °C and 55 °C (pH 8). The control (*E. coli* TG1 containing PUC18) values were subtracted from the sample values.

The esterolytic activity of *E. coli* TG1 containing pVS120 at different pH is shown in Figure 5. The highest activity was seen at pH 8. A reduction of activity was detected at pH 7, and at pH less than that the enzyme activity was only 20% of the maximum. Some enzyme activity was still seen at pH 10, but none at pH 11. Reduction of the enzyme activity below pH 7 may indicate that this enzyme has no relevance to the aroma production in Swiss-like cheese. The pH of this type of cheese during the

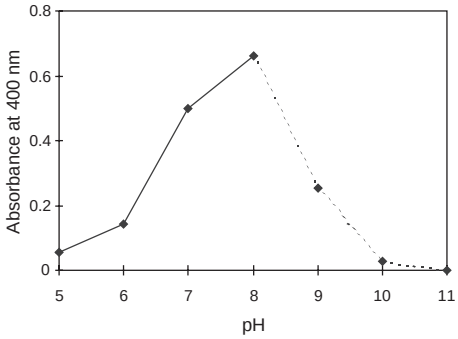

Figure 5. Effect of pH on the esterolytic activity of *E. coli* TG1 containing pVS120. Paranitrophenyl butyrate was used as a substrate at 20 ºC. Assays at pH 5–8 were performed in phosphate buffer and at pH 9 in boric acid-citric acid buffer (dash line).

ripening period is about pH 5.6–5.8. However, the protecting effect of milk components in cheese may change the optimum seen in this experiment.

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REFERENCES

[1] Atlas R.M., Tributyrin agar, in: Atlas R.M., (Ed.), Handbook of microbiological media for the examination of food, CRC Press Inc., Boca Raton, USA, 1995, pp. 257–258.

[2] Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., Struhl K., Current protocols in molecular biology, John Wiley & Sons, New York, USA, 1987.

[3] Brenner S., The molecular evolution of genes and proteins: a tail of two serines, Nature 334 (1988) 528.

[4] Castillo I., Requena T., Fernandez de Palencia P., Fontecha J., Gobbetti M., Isolation and characterization of an intracellular esterase from *Lactobacillus casei* subsp. *casei* IFPL731, J. Appl. Microbiol. 86 (1999) 653–659.

[5] Cousin M.A., Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review, J. Food Protect. 45 (1982) 172–207.

[6] Cummins C.S., Johnson J.L., Genus Propionibacterium, in: Sneath P.H.A., Mair N.S., Sharpe M.E., Holt J.G., (Ed.), Bergey´s manual of Systematic Bacteriology, Vol. 2, Williams and Wilkins, Baltimore, USA, 1984, pp. 1346–1353.

[7] Cygler M., Schrag J.D., Sussman J.L., Harel M., Silman I., Gentry M.K., Doctor B.P., Relationship between sequence conservation and three dimensional structure in a large family of esterases, lipases, and related proteins, Protein Sci. 2 (1993) 366–382.

[8] Drablos F., Petersen S., Identification of conserved residues in family of esterase and lipase sequences, Meth. Enzymol. 284 (1997) 28–61.

[9] Dupuis C., Corre C., Boyaval P., Lipase and esterase activities *of Propionibacterium freudenreichii* subsp. *freudenreichii*, Appl. Environ. Microbiol. 59 (1993) 4004–4009.

[10] Eichler K., Bourgis F., Buchet A., Kleber H-P., Mandrand-Berthelot M-A., Molecular characterization of the *cai* operon necessary for carnitine metabolism in *Escherichia coli*, Mol. Microbiol. 13 (1994) 775–786.

[11] El-Soda M., Ziada N., El-Shafei H.K., Esterolytic activity of *Propionibacterium* strains, Microbiol. Alim. Nutr. 11 (1993) 377–382.

[12] Fenster K.M., Parkin K.L., Steele J.L., Characterization of an arylesterase from *Lactobacillus helveticus* CNRZ32, J. Appl. Microbiol. 88 (2000) 572–583.

[13] Fernandez L., Beerhuyzen M.M., Brown J., Siezen R.J., Coolbear T., Holland R., Kuipers O.P., Cloning, characterization, controlled overexpression, and inactivation of the major tributyrin esterase gene of *Lactococcus lactis,* Appl. Environ. Microbiol. 66 (2000) 1360–1368.

[14] Gibson T.J., Studies on the Epstein-Barr virus genome, Ph.D. thesis (1984), Cambridge University.

[15] Gobbetti M., Fox P.E., Stepaniak L., Isolation and characterization of tributyrin esterase from *Lactobacillus plantarum* 2739, J. Dairy Sci. 80 (1997) 3099–3106.

[16] Gobbetti M., Smacchi E., Corsetti A., Purification and characterization of a cell surface-associated esterase from *Lactobacillus fermentum* DT41, Int. Dairy J. 7 (1997) 13–21.

[17] Hashimoto Y.M., Yamashita M., Murooka Y., The *Propionibacterium freudenreichii hem*YHBXRL gene cluster, which encodes enzymes and a regulator involved in the biosynthetic pathway from glutamate to protoheme, Appl. Microbiol. Biotechnol. 47 (1997) 385–392.

[18] Ikeda Y., Dabrowski C., Tanaka K., Separation and properties of five distinct acyl-CoA dehydrogenases from rat liver mitochondria, J. Biol. Chem 258 (1983) 1066–1076.

[19] Ladror U.S., Gollapudi L., Tripathi R.L., Latshaw S.P., Kemp R.G., Cloning, sequencing, and expression of pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii*, J. Biol. Chem. 266 (1991) 16550–16555.

[20] Malik A.C., Reinbold H.W., Vedamuthu E.R., An evaluation of the taxonomy of *Propionibacterium*, Can. J. Microbiol. 14 (1968) 1185–1191.

[21] Marsh E.N., McKie N., Davis N.K., Leadlay P.F., Cloning and structural characterization of the genes coding for adenosylcobalamin-dependent methylmalonyl-CoA mutase from *Propionibacteriun shermanii,* Biochem. J. 260 (1989) 345–352.

[22] Matsubara Y., Indo Y., Naito E., Ozasa H., Glassberg R., Volcley J., Ikeda Y., Kraus J., Tanaka K., Molecular cloning and nucleotide sequence of cDNAs encoding the precursors of rat long chain acyl-coenzyme A, short chain acyl-coenzyme A, and isovaleryl-coenzyme A dehydrogenases. Sequence homology of four enzymes of the acyl-CoA dehydrogenase family, J. Biol. Chem 264 (1989) 16321–16331.

[23] McKay D.B., Jennings M.P., Godfrey E.A., MacRae I.C., Rogers P.J., Beacham I.R., Molecular analysis of an esterase-encoding gene from a lipolytic psychrotrophic pseudomonad, J. Gen. Microbiol. 138 (1992) 701–708.

[24] Miskin J.E., Farrell A.M., Cunliffe W.J., Holland K.T., *Propionibacterium acnes*, a resident of lipid rich human skin, produces a 33 kDa extracellular lipase encoded by *geh*A, Microbiology 143 (1997) 1745–1755.

[25] Murakami K., Hashimoto Y., Murooka Y., Cloning and characterization of the gene encoding glutamate 1-semialdehyde 2,1-aminomutase, which is involved in δ-aminolevulinic acid synthesis in *Propionibacterium freudenreichii*, Appl. Environ. Microbiol. 59 (1993) 347–350.

[26] Norrander J., Kempe T., Messing J., Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis, Gene 26 (1983) 101–106.

[27] Oterholm A., Ordal Z.J., Witter L.D., Glycerol ester hydrolase activity of *Propionibacterium shermanii*, J. Dairy Sci. 53 (1970) 592–593.

[28] Paulsen P.V., Kowalewska J., Hammond E.G., Glatz B.A., Role of microflora in production of free fatty acids and flavor in Swiss cheese, J. Dairy Sci. 63 (1980) 912–918.

[29] Sattler I., Roessner C.A., Stolowich N.J., Hardin S.H., Harris-Haller L.W., Yokubaitis N.T., Murooka Y., Hashimoto Y., Scott I., Cloning, sequencing, and expression of the uroporphyrinogen III methyltransferase *cobA* gene of *Propionibacterium freudenreichii* (*shermanii*), J. Bacteriol. 177 (1995) 1564–1569.

[30] Varmanen P., Rantanen T., Palva A., Tynkkynen S., Cloning and characterization of a prolinase gene (*pepR*) from *Lactobacillus rhamnosus*, Appl. Environ. Microbiol. 64 (1998) 1831–1836.