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The Bioengineering and Industrial Applications of Bacterial Alkaline Proteases: the Case of SAPB and KERAB

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1. Introduction

Enzymes have long been used as alternatives to chemicals to improve the efficiency and cost-effectiveness of a wide range of industrial systems and processes. They are currently used in basic and applied arenas of research as well as in a wide range of product design and manufacturing processes, such as those pertaining to the food, beverage, pharmaceutical, detergent, leather processing, and peptide synthesis industries (Gupta et al., 2002). Of particular interest to the aims of the present work, proteases have often been reported to constitute a resourceful class of enzymes with promising industrial applications. According to recent estimates, these enzymes account for nearly 65% of total worldwide enzyme sales (Anonyme, 2007; Rao et al., 1998). They are widely distributed in nature and play a vital role in life processes. They are particularly known for their capacity to hydrolyze peptide bonds in aqueous environments and to synthesize peptide bonds in non-aqueous biocatalysis.

Proteases have been employed in a wide array of applications for many years with satisfactory results. They constitute a large family of enzymes present in a wide range of living organisms, such as plants, animals and microorganisms. In biotechnologically oriented systems and processes, however, proteases from microbial origins have often been reported to have distinct advantages when compared to plant or animal proteases, particularly because they possess almost all the characteristics desired for biotechnological applications. Among these biocatalysts, high-alkaline proteases, which alone account for about 40% of the total worldwide enzyme sales (Kirk et al., 2002), proved particularly suitable for industrial use. This is mainly due to their high stability and activity under harsh conditions.

Nowadays, the use of alkaline protease-based detergents is preferred over the conventional synthetic ones. This is partly because of their better cleaning properties, higher performance efficiency at lower washing temperature, and safer dirt removal conditions (Gupta et al., 2002). Typically, a detergent protease needs to be active, stable, and compatible with the alkaline environment encountered under harsh washing conditions: pH 9 - 11, temperature of 20 - 60°C, as well as high concentrations of salt, bleach, and surfactant. Some of the alkaline proteases that are particularly preferred in contemporary detergent formulations include Savinase™ (Subtilisin 309), Subtilisin Novo (BPN'), Alcalase™ (Subtilisin Carlsberg; SC), Maxacal™ (Novozymes A/S, Denmark), BLAP S^b (Henkel, Germany) and Properase™ (Genecor Int. USA). They are often reported to be stable at conditions of elevated temperatures and pH. Most of them have, however, been criticized for their limited efficiency in the presence of liquid or solid laundry detergents wherein their stability decreases (Beg and Gupta, 2003; Maurer, 2004). Therefore, the search for and screening of alternative microorganisms that produce detergent-stable enzymes and preserve their high activity and stability at extreme conditions would be highly desired, particularly within the framework of the persistent aspirations that consumers, industrialists and, by extension, researchers, have towards improved laundry detergents with powerful, safe and healthy cleansing abilities.

Various alkaline proteases have been reported to constitute appropriate additives for a variety of detergent, laundry and cleansing supplies as well as other leather processing, dyeing, and finishing applications. Keratinases are a group of mostly extracellular serine-proteases that have often been reported for their excellent potency to degrade keratins, a group of fibrous, insoluble and abundant structural proteins that constitute the major components of structures growing from the skin of vertebrates, such as hair, wool, nails, hooves, horns and feather quills. In fact, due to their high degree of cross-linking to disulphide bonds, hydrogen bonds, and hydrophobic interactions, these proteins show high stability and resistance to proteolytic hydrolysis (Coulombe and Omary, 2002).

Large amounts of keratin containing wastes are discharged every year from poultry, leather and meat processing industries. Current estimates indicate that the global annual discharge of feather from the poultry processing industry alone reaches millions of tons (C.A.S.T., 1995; Freeman et al., 2009). This keratinous poultry waste is degraded very slowly in nature and is, therefore, considered hazardous to the environment. Seeing that keratinous waste represents a valuable source for proteins and amino acids, several steam pressure and chemical treatment processes have been developed to convert feathers into feather meal for animals (Hess and FitzGerald, 2007). These physico-chemical conversion methods have, nevertheless, been reported to involve costly treatments under harsh temperature and pressure conditions that result in the loss of essential amino acids (Onifade et al., 1998). Alternatively, feather biodegradation processes have been proposed as viable substitutes (Ignatova et al., 1999; Xie et al., 2010).

Keratinolytic microorganisms can be employed in the manufacture of nutritious, cost-effective, environmentally safe feather meal for poultry, as well as in the enhancement of drug delivery, hydrolysis of prions, construction of biodegradable films, and production of biofuels (Brandelli et al., 2010). Additionally, these keratinolytic enzymes have a variety of current and potential applications in a wide range of biotechnological processes that involve keratin hydrolysis, including the enzymatic dehairing and catalysis for leather and cosmetic industries, the breaking down of recalcitrant matter for the laundry and detergent industries, the slowing down of nitrogen release for fertilizer and pesticide industries, and the production of biohydrogen and rare amino acids for animal feed and foodstuff industries (Bertsch and Coello, 2005).

Several microorganisms that possess keratinolytic activity have been reported to accede to the biodegradation of keratin waste by secreting keratinolytic peptidases into the culture medium and to offer valuable tools for the development of efficient and cost-effective keratin waste bioconversion methods (Onifade et al., 1998). In this respect, various keratinases have been purified from different microorganisms, namely fungi, such as *Microsporum* (Essien et al., 2009) and *Chryseobacterium indologenes* TKU014 (Wang et al., 2008), and bacteria, such as *Bacillus* (Pillai and Archana, 2008; Radha and Gunasekaran, 2008) and *Streptomyces* (Syed et al., 2009; Tatineni et al., 2008). As corresponds to their habitat, these bacteria are nutritionally quite versatile, and most of them produce extracellular hydrolytic enzymes that permit the use of high-molecular-weight biopolymers, such as proteins, polysaccharides, fats, and a variety of other substrates (Gupta et al., 1995). Among these enzymes, several serine peptidases have so far been isolated, purified, and characterized from various species, such as *S. griseus* (Awad et al., 1972; Johnson and Smillie, 1974), *S. fradiae* (Kitadokoro et al., 1994), *S. thermoviolaceus* SD8 (Chitte et al., 1999), and *S. graminofaciens* (Szabo et al., 2000).

Despite this large flow of data on keratinases, however, little information has so far been reported on the characterization and purification of keratinases from *Streptomyces*. Moreover, and particularly due to the relatively poor levels of stability and catalytic activity obtained for the *Streptomyces* enzymes so far investigated under the specific operational conditions required by current industrial applications, namely high temperature and pH values, as well as the presence of detergents or non-aqueous solvent, their practical application still remained very limited. Accordingly, the isolation and screening of new keratinolytically active *Streptomyces* strains from natural habitats could open new pathways for the discovery and use of novel keratinases.

The present chapter aim to provide an overview on the current quest for novel natural bacterial alkaline proteases with special emphasis on the purification and characterization of two enzymes, namely SAPB and KERAB, from isolated alkaline proteinase and keratinase producing microbial strains, whose promising properties and attributes are likely to open new pathways in current and future research and new possibilities for the improvement of current detergent formulations and leather processing industries. In fact, both SAPB and KERAB showed valuable operational characteristics that made them strong potential candidates for future application as additives in biotechnological applications and processes, particularly in detergent formulations and in dehairing during leather processing. They also showed relatively high stability in the presence of organic solvents, a feature which is highly desired in applications involving the biocatalysis of non-aqueous peptides. Accordingly, this chapter intends to report on the screening, identification, and phylogenetic analysis of the *Bacillus pumilus* strain CBS producing SAPB and the *Streptomyces* sp. strain AB1 producing KERAB. It also aims to describe the laundry detergent compatibility and high dehairing capacity of both enzymes, and to report on the ability of each strain or enzyme (SAPB or KERAB) alone to accomplish the whole keratin-degradation process of various keratinacious biowastes.

2. Screening and identification of alkaline proteinase and keratinase producing microbes

The isolation and screening of micro-organisms from naturally occurring alkaline habitats and keratinacious biowaste is likely to help identify potential microbial strains capable of

producing active and stable enzymes that can resist the aforementioned harsh substances and conditions present in detergent formulations and leather dehairing processes.

2.1 Screening of alkaline protease and keratinase producing strains

A recent work by the authors (Jaouadi et al., 2009; Badis et al., 2009) involved the screening of about 125 bacterial strains (Bacilli and Actinomyces), originating from a collection of bacterial strains at the CBS and other strains that were previously isolated from surface soil samples at the Mitidja plain, North of Algeria (Badis et al., 2010), for protease and keratinase activities. Based on the ratio of the diameter of the clear zone (onto skimmed milk or keratin-containing medium agar plates at pH 9.) and that of the colony, only 24 isolates, which exhibited the highest ratio (> 3 mm), were selected for further assays pertaining to protease or keratinase production in liquid media. The two bacterial strains that displayed the highest extracellular protease and keratinase activity were termed as strain CBS (from the CBS bacterial strain collection) and strain AB1 (from Algerian soil samples) and retained for all subsequent experimental assays.

2.2 Identification and molecular phylogeny of the microorganisms

The two newly isolated bacterial strains, CBS and AB1, were submitted to identification and typing by molecular and catabolic techniques. The data from the morphological, biochemical and physiological characterization tests, performed on the isolates in accordance with the methods described in the Bergey's Manual of Systematic Bacteriology, showed that the CBS and AB1 strains appeared in a bacilli and filamentous form, respectively, that are aerobic, endospore-forming, Gram-positive, catalase+, oxydase+ and motile rod-shaped. The findings from API 50 CH gallery tests revealed that the CBS isolate metabolized L -arabinose, D-tagatose, ribose, and mannitol in addition to several other simple sugars. The AB1 strain, on the other hand, could use galactose, sucrose, maltose, cellobiose, fucose, raffinose, D-xylose, L-arabinose, and D-ribose, but not lactose, starch, L-rhamnose, erythritol, adonitol, and inositol. The results from API ZYM tests revealed that strain AB1 also exhibited alkaline phosphatase, esterase lipase (C8), leucine arylamidase and valine arylamidase activities, but no lipase (C14), trypsin, α-chymotrypsin, *N*-acetyl-β-lucosamidase, β -glucuronidase, α-mannosidase, and α-fucosidase ones. Taken together, the data obtained with regard to the physiological and biochemical properties of the two isolates strongly confirmed that the strains CBS and AB1 belonged to the *Bacillus* and *Streptomyces* genera, respectively.

A molecular approach was used to establish further support for the identification of the CBS and AB1 isolates. Two 16S rRNA gene fragments, namely 1,497 bp (Jaouadi et al., 2009) and 1541 bp (Jaouadi et al., 2010a), were amplified from the genomic DNA of the CBS and AB1 isolates, respectively, and then cloned and sequenced on both strands. The 16S rRNA gene sequences obtained were subjected to GenBank BLAST search analyses, which yielded strong homologies of up to 98 and 99% with those of several cultivated strains of *Bacillus* and *Streptomyces*, respectively. The nearest *Bacillus* and *Streptomyces* strains identified by the BLAST analysis were the *Bacillus pumilus*, with the accession numbers of DQ988522, AM292995, AY548955, AB195283, and EF173329, and the *Streptomyces rochei* strains of A-1 (GQ392058) and NRRL B-1559 (EF626598) as well as the *Streptomyces* sp. Strain B5W22-2 (EF114310), respectively. Those sequences were imported into the ARB and MEGA software packages, respectively, and then aligned. After that, the phylogenetic trees were constructed

using neighbour-joining methods and Jukes-Cantor distance matrices (Fig. 1). Phylogenetic analyses confirmed that the CBS and AB1 strains were closely related to the five isolated *Bacillus* and three isolated *Streptomyces* strains mentioned earlier. In conclusion, all the results obtained strongly supported the assignment of the CBS and AB1 isolates to the *Bacillus pumilus* strain CBS and *Streptomyces* sp. strain AB1, respectively.

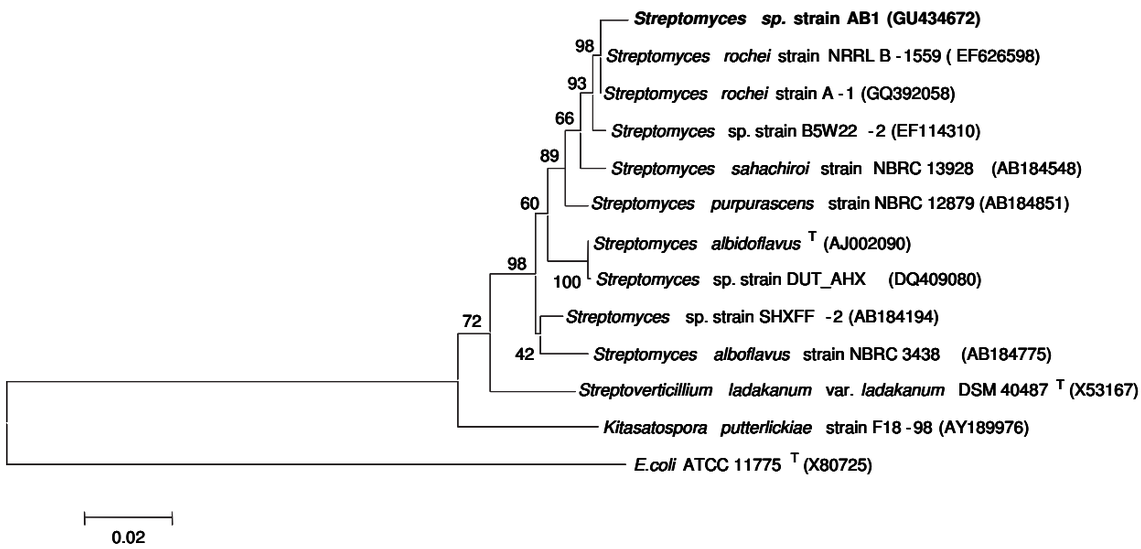


Fig. 1. Example of the phylogenetic tree of *Streptomyces sp.* strain AB1. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.1. Reference type-strain organisms are included and sequence accession numbers are given in parentheses. Bootstrap values, expressed as percentage of 100 replications, are shown in branching points and bar indicated 2 substitutions per 100 nt. The out-group used in the analysis, *E. coli* (X80725), was chosen arbitrarily.

3. Production, purification and biochemical characterization of SAPB and KERAB enzymes

3.1 SAPB and KERAB production

Different carbon and nitrogen sources and trace elements were assayed to optimize the culture growth conditions for the production of the enzymes. In the medium containing (g/l): gelatin 10, yeast extract 5, $CaCl_2$ 1, K_2HPO_4 1, and KH_2PO_4 1, the addition of 0.1% (v/v) trace elements [composed of (g/l): $ZnCl_2$, 0.4; $FeSO_4 \cdot 7H_2O$, 2; H_3BO_3, 0.065; and $MoNa_2O_4 \cdot 2H_2O$, 0.135] at pH 10 was noted to bring about a significant enhancement of 1.32 folds in SAPB production, which reached 6,500 U/ml under the optimal conditions used (pH 10.6 and 65°C), after 24 h of incubation at 37°C and 250 rpm (Jaouadi et al., 2009). In medium containing trace salts with feather as carbon and nitrogen source (g/l): NaCl, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 0.5; KCl, 0.1; $MgSO_4 \cdot 7H_2O$, 1; and chicken feather meal, 10; at pH 9, KERAB production was observed to undergo a significant improvement, reaching a maximum of 9,500 U/ml under the optimal conditions used (pH 11.5 and 75°C) after 96 h of incubation at 30°C and 200 rpm (Jaouadi et al., 2010a). Under these particular conditions, the production of the SAPB and KERAB enzymes started after a 6- and 10-h lag phase,

respectively. These productions were then noted to increase exponentially and concomitantly with the increase of cellular growth and to reach the maxima within 24 h of cultivation for SAPB (Fig. 2) and 96 h for KERAB (data not shown).

Compared to the production yields obtained in flask cultivations, the use of a 7-litre fermentor containing the optimized medium after 24-h cultivation at 37°C, an aeration of 1.5 vvm, and an agitation of 600 rpm was noted to improve SAPB production by about 4-folds. It is worth noting here that the cell densities obtained in both cases (Rotary flask and fermentor) were almost the same (about O.D. = 10.9). Based on this particular finding, it was possible to infer that the improvement of enzyme production was related not only to the cell's growth but also to the stability of fermentation parameters (pH and pO_2).

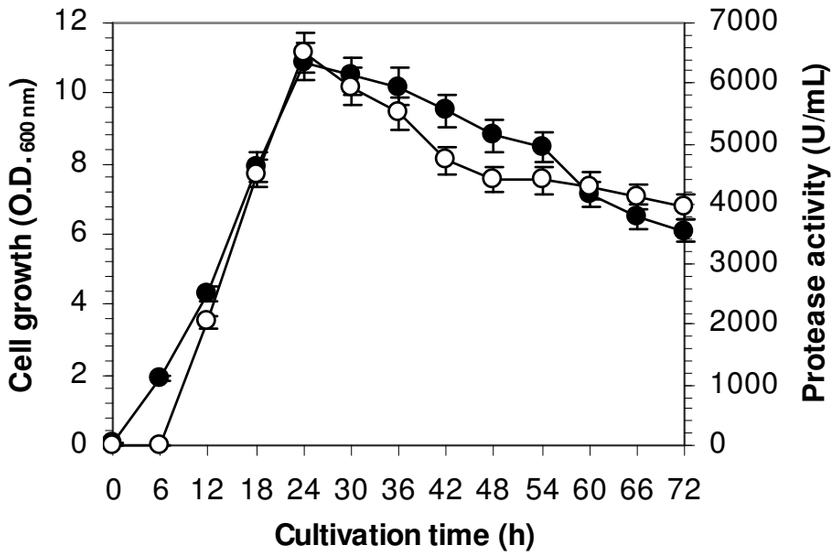


Fig. 2. Time course of *B. pumilus* strain CBS cell growth (●) and SAPB production (○). The culture was carried out under the submerged shaking flask conditions at 37°C for 72 h with an agitation rate of 250 rpm in broth medium containing (g/l): gelatin 10, yeast extract 5, $CaCl_2$ 1, K_2HPO_4 1, KH_2PO_4 1, and trace elements 0.1% (v/v) at pH 10. Cell growth was monitored by measuring the O.D. at 600 nm.

3.2 SAPB and KERAB purification and characterization

The purification protocols used for the purification of each enzyme were conducted at temperatures not exceeding 4°C. Five-hundred ml of 24 h and 96 h cultures of *B. pumilus* strain CBS and *Streptomyces* sp. strain AB1, respectively, were centrifuged to remove microbial cells. Ammonium sulfate was added to each supernatant to a final concentration of 270 g/l. In the case of SAPB, the precipitate formed was collected by centrifugation, dissolved in a minimum amount of 50 mM Tris-HCl (pH 7.5) supplemented with 2 mM $CaCl_2$ and 0.05% Triton X-100 (Buffer A). In the case of KERAB, the precipitate was suspended in 50 mM bicarbonate-NaOH buffer and supplemented with 5 mM $MgSO_4$ at pH 11.5 (Buffer B) containing 10 mM NaCl (Buffer C), and then dialyzed overnight against repeated changes of the buffer A and C, respectively.

Purification to homogeneity was achieved for SAPB by HPLC using Shodex Protein WK 802-5 column. The analysis indicated that enzyme achieved a degree of purity that was

about 38-fold greater than that of the crude extract. Under the optimal assay conditions used, the purified enzyme preparation exhibited a yield of about 12% with a specific activity of 25,500 U/mg (Jaouadi et al., 2008). As far as KERAB was concerned, the insoluble material was then removed by centrifugation. The supernatant obtained was incubated for 1 h at 50°C and insoluble material was removed by centrifugation. The supernatant was loaded on a Sephacryl S-200 column equilibrated with buffer B. The elution of protease was performed with the same buffer. The fractions containing keratinase activity were then pooled and applied to a Q-Sepharose column equilibrated in buffer D. The column was rinsed with 500 ml of the same buffer and the adsorbed material was eluted with a linear NaCl gradient. At the final purification step, Keratinase activity was eluted between 0.15 and 0.3 M NaCl. The purity of the enzyme was estimated to be about 86-fold greater than that of the crude extract. The purified enzyme preparation contained about 24% of the total activity of the crude enzyme and had a specific activity of 67,000 U/mg (Jaouadi et al., 2010a). These preparations were homogeneous enzymes with high purity as they exhibited single protein bands on native PAGE and unique elution symmetrical peaks on gel filtration chromatography.

For determination of their molecular weight, enzyme preparations were treated with 1 mM PMSF prior to electrophoresis to inhibit possible autolysis during electrophoresis. Electrophoresis under denaturing conditions (SDS-PAGE) also revealed single bands with molecular masses estimated as 34 kDa for SAPB (Jaouadi et al., 2008) and 30 kDa for KERAB (Jaouadi et al., 2010a). The exact molecular masses obtained for the purified SAPB and KERAB were confirmed by MALDI-TOF mass spectrometry as being 34598.19 and 29850.17 Da, respectively. Zymogram activity staining also revealed two clear zones of proteolytic activity at 34 and 30 kDa for the SAPB and KERAB, respectively. These observations indicated that SAPB extracted from the newly isolated bacterium *B. pumilus* CBS was a monomeric holoenzyme comparable to those previously reported for other proteases from *B. pumilus* strains (Han and Damodaran, 1998; Huang et al., 2003; Kumar, 2002; Miyaji et al., 2006; Yasuda et al., 1999). They also showed that KERAB was a monomeric protein comparable to those previously reported for other proteases from *Streptomyces* strains (Syed et al., 2009; Tatineni et al., 2008).

The molecular mass of SAPB determined by SDS-PAGE (~ 34000 Da) and conducted by MALDI-TOF mass spectrometry (34598.19 Da) were not close to that calculated from the primary sequence of the mature polypeptide (27789 Da), which strongly suggested that the protein underwent noteworthy post-translational changes that were presumably pertaining to glycosylation. Similar differences between experimental and theoretical determinations were previously observed for several *B. pumilus* proteases, including those from *B. pumilus* TYO-67 (Yasuda et al., 1999), *B. pumilus* UN-31-C-42 (Huang et al., 2003), and *B. pumilus* MS-1 (Miyaji et al., 2006).

3.3 Physico-chemical and kinetic properties of SAPB and KERAB

Phenylmethanesulfonyl fluoride (PMSF) and diiodopropyl fluorophosphates (DIFP) were noted to strongly inhibit SAPB and KERAB, which indicated that both enzymes belonged to the serine proteases family. While the optimal pH and temperature values of 10.6 and 65°C were determined for SAPB using casein as a substrate, those obtained for KERAB were 11.5 and 75°C with keratin azure as substrate. The thermoactivity and thermostability of KERAB were also demonstrated to be enhanced in the presence of 5 mM Mg^{2+} against 2 mM Ca^{2+} for

SAPB. One of the distinguishing properties of SAPB was its catalytic efficiency (k_{cat}/K_m) which was 4.77, 2.73, and 2.11 times higher than those of Subtilisin Carlsberg, Subtilisin BPN', and Subtilisin 309, respectively. The catalytic efficiency of KERAB was higher than those of SAPB, nattokinase and subtilisin Carlsberg.

3.4 Substrate specificity of SAPB and KERAB

The activity of the purified SAPB and KERAB enzymes towards various natural and modified protein substrates is summarized in Table 1. Among the proteinaceous substrates tested, casein and keratin were most efficiently hydrolyzed by SAPB and KERAB, respectively. When SAPB and KERAB activities against casein and keratin were taken as 100%, the hydrolysis rates of gelatine and casein were 95 and 92%, respectively. Poor BSA hydrolysis rates were, however, noted in both cases. A similarly low hydrolysis level was also observed with gluten and egg albumin. Using modified proteins as substrates, the highest activities observed for SAPB and KERAB were with azocasein and keratin azure, respectively. Previous reports also showed that alkaline serine proteases from *B. stearotermophilus* FI (Rahman et al., 1994) and *Bacillus pumilus* A1 (Fakhfakh-Zouari et al., 2010) exhibited highest activities towards casein and keratin, respectively. Interestingly, no collagenase activities were detected for SAPB and KERAB on collagen types I and II, which suggests the potential utility of both enzymes for hair removal in the leather industry.

The cleavage specificities of SAPB and KERAB toward various oligopeptidyl and ester substrates were also investigated. The findings revealed that SAPB exhibited both esterase and amidase activities on oligopeptides, with Tyr or Phe at position P_1 (the amino acid residue at the N-terminal side of the scissile peptide bond). This included *N*-benzol-L-arginine ethyl ester (BTEE) or *N*-acetyl-L-tyrosine ethyl ester monohydrate (ATEE) and *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (AAPF), which are specific substrates for chymotrypsin-like proteases (DelMar et al., 1979; Walsh, 1970). In fact, however, the activity of SAPB on AAPF did not necessarily mean that it was a chymotrypsin-like enzyme. Firstly, most of the microbial members of the Subtilisin family are reported to have specificity that is somewhat similar to that of chymotrypsin (Rawlings and Barrett, 1977). Moreover, SAPB was not observed to show sensitivity to *N*α-p-tosyl L-phenylalanine chloromethyl ketone (TPCK), which is an inhibitor of chymotrypsin-like enzymes (Schoellman and Shaw, 1963). Last but not least, SAPB showed neither esterase nor amidase activity on synthetic substrates with P_1 = Arg, such as *N*-benzol-L-arginine ethyl ester (BAEE) and *a*-benzoyl-L-tyrosine *p*-nitroanilide (BAPNA), which are substrates for trypsin-like proteases (Rick, 1995). In contrast, the purified KERAB exhibited esterase and amidase activities on BAEE and BAPNA, but not on BTEE and ATEE.

In the same way, the purified KERAB was noted to exhibit a preference for aromatic and hydrophobic amino acid residues, such as Phe, Leu, Ala, and Val, at the carboxyl side of the splitting point in the P1 position. KERAB was, therefore, active against leucine peptide bonds. When Suc-$(Ala)_n$-*p*NA was used as the synthetic oligopeptide substrate, a minimum length of three residues was necessary for hydrolysis. Enzymatic activity was observed to depend mainly on secondary enzyme substrate contacts with amino acid residues (P2, P3, etc.) more distant from the scissile bond, as illustrated by the differences observed between the kinetic parameters of Suc-$(Ala)_2$-Val-*p*NA and those of Suc-Tyr-Leu-Val-*p*NA. The highest hydrolysis levels achieved by KERAB and SAPB were 100% for AAPF and Suc-Tyr-Leu-Val-*p*NA, respectively.

Substrate	Concentration	Relative activity (%)*	
		SAPB	KERAB
Natural protein[a]			
Keratin	10 g/l	65 ± 1.4	100 ± 3.0
Casein	20 g/l	100 ± 2.5	92 ± 2.0
Gelatine	20 g/l	95 ± 2.4	79 ± 1.4
BSA	20 g/l	52 ± 1.3	66 ± 1.4
Albumin (egg)	20 g/l	15 ± 0.7	26 ± 0.9
Gluten (wheat)	20 g/l	20 ± 0.8	11 ± 0.8
Modified protein[b]			
Keratin azure	10 g/l	63 ± 1.4	100 ± 3.0
Azo-casein	20 g/l	100 ± 2.5	94 ± 2.5
Collagen type I[c]	1 mg/ml	0 ± 0.0	0 ± 0.0
Collagen type II[c]	1 mg/ml	0 ± 0.0	0 ± 0.0
Ester[d]			
BAEE	4 mM	0 ± 0.0	100 ± 3.0
BTEE	3 mM	71 ± 1.4	10 ± 1.5
ATEE	3 mM	100 ± 2.5	20 ± 0.9
Synthetic peptide[e]			
Suc-Tyr-Leu-Val-*p*NA	2 mM	30 ± 1.1	100 ± 3.0
Suc-$(Ala)_2$-Pro-Phe-*p*NA	3 mM	100 ± 2.5	17 ± 0.9
Suc-$(Ala)_2$-Pro-Leu-*p*NA	3 mM	45 ± 1.3	13 ± 0.8
Suc-$(Ala)_2$-Val-Ala-*p*NA	3 mM	39 ± 1.2	50 ± 1.8
Suc-$(Ala)_2$-Val-*p*NA	3 mM	25 ± 0.9	56 ± 2.0
Suc-$(Ala)_3$-*p*NA	2 mM	10 ± 0.4	67 ± 2.2
Suc-$(Ala)_2$-Phe-*p*NA	2 mM	17 ± 0.5	89 ± 2.5
BAPNA	2 mM	0 ± 0.0	66 ± 1.3

[a]The activity of these natural protein substrates were assessed by measuring absorbance at 660 nm following the previously reported Folin-Ciocalteu method (Jaouadi et al., 2010b).
[b]The activity of these modified protein substrates were determined by measuring absorbance at 440 nm following the method of Riffel and Brandelli, (2002).
[c]The collagenolytic activity was determined by measuring absorbance at 490 nm as described in the protocol of Sigma Co.
[d]The esterase and amidase activities of these substrates were determined by measuring absorbance at 253 nm as described in the method of Walsh (Walsh, 1970).
[e]The activity of these synthetic oligopeptide substrates was determined by measuring absorbance at 410 nm according to the method of DelMar et al., (1979).
*Values represent the mean of four replicates and standard errors are reported.

Table 1. Substrate specificity of SAPB and KERAB.

4. Molecular cloning of *sapB* gene and engineering of more efficient SAPB mutant enzymes

The *sap*B gene encoding SAPB was cloned, sequenced, and over-expressed in *Escherichia coli*. The purified recombinant enzyme, called rSAPB, exhibited the same biochemical properties of the native enzyme (Jaouadi and Bejar, 2008). An additional study by the authors further investigated the implications of five amino acid residues (L31, T33, N99, F159, and G182) on the pH and temperature behavior as well as kinetic parameters of the enzyme using site-directed mutagenesis and 3D-modeling approaches (Jaouadi et al., 2010b). Seven more efficient SAPB mutant enzymes, particularly L31I/T33S/N99Y, were generated. The latter had an optimal of pH of 12 and an optimal temperature of 70°C. It was also noted to exhibit a high specific activity that was approximately 2-fold higher than that of the wild-type enzyme and a prominent increase in its k_{cat}/K_m value that was 42-fold higher than that of the wild-type enzyme.

5. Potential and prospects for SAPB and KERAB in detergent formulations

5.1 Effect of detergents on the activity and stability of SAPB and KERAB

With the aim of evaluating the performance of the purified proteases in real life-like detergents, SAPB and KERAB were pre-incubated at 40°C and in the presence of several commercially available laboratory non-ionic surfactants, denaturing agents or anionic surfactants, and bleach agents for 24 and 72 h, respectively. The residual activity was determined at pH 10.6 and 65°C (for SAPB) and pH 11.5 and 75°C (for KERAB). The findings revealed that the SAPB enzyme exhibited high stability at 10% of oxidizing agents (Tween 60 or Triton X-100) as well as against strong anionic surfactants, particularly sodium dodecyl sulphate(SDS) and linear alkylbenzene sulfonate (LAS) (Jaouadi et al., 2008). In fact, SAPB retained its activity upon treatment with 0.8% SDS and 0.5% LAS. In addition, 80 and 65% residual activity were obtained after incubation with 1.5% SDS and 1% LAS, respectively. The SAPB and KERAB enzymes were also highly stable against bleaching agents for they retained 110 and 115% of their initial activity after treatment with 15% hydrogen peroxide, respectively. This is an important behaviour of SAPB and KERAB because oxidant-, surfactant-, and bleach-stable wild-type enzymes are rarely reported.

By way of comparison, the alkaline protease from alkalophilic *Bacillus* sp. JB-99 lost 25% activity during treatment with 0.5% SDS for only 1 h of incubation at 40°C (Johnvesly and Naik, 2001) while two other alkaline proteases (FI and FII) from *Vibrio fluvialis* TKU005 were activated by 1% SDS (Wang et al., 2007b). The present native SAPB and KERAB enzymes showed inherent stability in the presence of high concentrations of detergent compounds, especially Tween 60 at 10%, SDS at 1.5%, and hydrogen peroxide at 15%. In addition, their enzymatic activity and stability were observed to improve in the presence of high concentrations of 1% perfume and anti-redeposition agents, particularly 100 mM Na_2-CMC, and of cationic (TTAB, CTAB) and zwitterionic (Zwittergent 3-12, CHAPS) detergent agents (Table 2). This stability is of interest since only few wild-type proteases have so far been reported to be oxidant, surfactant and bleach stable. These include those reported by Gupta et al., (1999) and Haddar et al., (2009). Bleach stability was also attained through protein engineering (Pillai and Archana, 2008; Radha and Gunasekaran, 2008). These findings suggest the potential strong candidacy of SAPB and KERAB for application as cleaning additives in detergent formulations to facilitate the release of proteinacious materials in tough stains caused by blood, chocolate, grime, milk, etc.

Detergent additive	Concentration	Relative activity (%)		Residual activity (%)	
		SAPB	KERAB	SAPB	KERAB
None	–	100 ± 2.5	100 ± 2.5	100 ± 2.5	100 ± 2.5
H_2O_2	15%	140 ± 3.7	155 ± 3.8	110 ± 2.6	115 ± 2.6
Sodium perborate	2% (w/v)	85 ± 2.2	110 ± 2.6	55 ± 2.0	85 ± 2.2
SDS	1.5%	110 ± 2.6	125 ± 3.0	80 ± 2.2	109 ± 2.6
LAS	1% (w/v)	79 ± 2.2	120 ± 3.2	65 ± 2.1	103 ± 2.5
Sulfobetaine	30 mM	105 ± 2.5	130 ± 3.3	90 ± 2.3	113 ± 2.6
Tween 40	5% (v/v)	111 ± 2.6	135 ± 3.4	101 ± 2.5	119 ± 2.8
Tween 60	10% (v/v)	120 ± 3.0	126 ± 3.3	105 ± 2.5	111 ± 2.6
Triton X-100	10% (v/v)	101 ± 2.5	132 ± 3.5	94 ± 2.3	112 ± 2.7
TAED	10% (w/v)	115 ± 2.8	128 ± 3.5	103 ± 2.5	117 ± 2.7
Na_2-CMC	5% (w/v)	109 ± 2.6	137 ± 3.5	101 ± 2.5	112 ± 2.6
Zeolithe	1% (w/v)	99 ± 2.5	100 ± 2.5	94 ± 2.3	95 ± 2.3
STPP	1% (w/v)	88 ± 2.3	90 ± 2.3	80 ± 2.2	82 ± 2.2
Perfume	1% (v/v)	115 ± 2.6	116 ± 2.6	103 ± 2.5	104 ± 2.5
Na_2-CO_3	100 mM	50 ± 2.0	113 ± 2.4	42 ± 1.8	100 ± 2.5
Zwittergent 3-12	10 mM	107 ± 2.5	116 ± 2.6	100 ± 2.5	109 ± 2.5
CHAPS	15 mM	121 ± 3.0	133 ± 3.1	106 ± 2.5	115 ± 2.6
CTAB	25 mM	104 ± 2.5	107 ± 2.3	95 ± 2.4	100 ± 2.5
TTAB	25 mM	99 ± 2.4	105 ± 2.6	90 ± 2.3	98 ± 2.3

Sulfobetaine: *N*-dodecyl-N-N'-dimethyl-3-ammonio-1-propane sulfonate; Tween: poly (oxyethylene) sorbitan monolaurate; Triton: octyphenolpoly (ethylene glycolether); TAED: tetraacetylethylenediamine; STPP: sodium tripolyphosphate; CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; CTAB: hexadecyltrimethylammonium bromide; TTAB: tetradecyl trimethylammonium bromides.

Table 2. Effect of various detergents on SAPB and KERAB activity and stability. The non-incubated enzymes were considered as 100%. The activity is expressed as a percentage of the activity level in the absence of additives. Values represent the mean of three replicates and standard errors are reported.

5.2 Compatibility of SAPB and KERAB enzymes with various commercial laundry detergents

To check the compatibility and stability of the alkaline proteases towards detergents, the enzymes were pre-incubated in the presence of various commercial laundry detergents of different compositions for 1 h at 40°C. The laundry detergents were diluted in tap water to a final concentration of 7 mg/ml to simulate washing conditions. The endogenous proteases were inactivated by incubating the diluted detergents for 1 h at 65°C, prior to the addition of the SAPB and KERAB enzymes, or the SB 309 commercial enzyme, which was used for comparison (Table 3). The findings showed that SAPB and KERAB were relatively more stable and compatible with some commercial liquid detergents than the commercial enzyme. In fact, while SB 309 retained 100, 85, 70 and 90% of its initial activity in the presence of Axion, Dinol, Nadhif, and Lav+, SAPB retained about 100, 95, 94, and 85% and KERAB about 87, 90, 75, and 95% of their initial activities, respectively. The SAPB and SB 309 enzymes were, however, less stable in the presence of Axion, where they were totally

active. Furthermore, SAPB and KERAB showed excellent stability and compatibility in the presence of some commercial solid detergents, namely OMO, New Det, and Skip, with SAPB retaining about 96, 82, and 69% of its initial activity, and KERAB about 88, 93, and 95%, respectively. SAPB and KERAB were, however, less stable in the presence of Ariel, retaining about 55 and 51% of their initial activities, respectively. Nevertheless, the compatibility and stability exhibited by SAPB and KERAB were much more significant than that of SB 309, which retained only 70, 68, and 84% of its initial activity in the presence of OMO, New Det, and Skip, respectively. Incubated in the same conditions in the presence of New Det, the NH1 protease was reported to retain 60% of its initial activity (Hadj-Ali et al., 2007) and, in the presence of Ariel, the VM10 (Venugopal and Saramma, 2006) and SSR1 (Singh et al., 2001) proteases were reported to retain only 42 and 37% of their initial activities, respectively. Overall, the results obtained clearly indicated the superior performance of SAPB and KERAB enzymes in detergents compared to currently commercialized or previously described proteases. A minor discordance was, however, reported as present with regards this performance, which was presumably correlated to the nature and concentration of the laundry detergent compounds used.

Laundry detergent (7 mg/ml)	**Relative activity (%)**			**Residual activity (%)**		
	SAPB	**KERAB**	**SB 309**	**SAPB**	**KERAB**	**SB 309**
None	100 ± 2.5	100 ± 2.5	100 ± 2.5	100 ± 2.5	100 ± 2.5	100 ± 2.5
Liquid detergent						
Dinol	95 ± 2.4	90 ± 2.2	85 ± 2.2	81 ± 1.4	80 ± 2.2	77 ± 2.0
Lav+	85 ± 2.2	95 ± 2.4	90 ± 2.2	75 ± 2.0	81 ± 2.2	75 ± 2.0
Nadhif	94 ± 2.4	75 ± 2.0	70 ± 2.0	77 ± 2.0	62 ± 1.8	60 ± 1.7
Axion	100 ± 2.5	87 ± 2.1	100 ± 2.5	91 ± 2.3	66 ± 1.9	85 ± 2.2
Solid detergent						
New Det	99 ± 2.5	94 ± 2.4	68 ± 2.0	82 ± 2.2	93 ± 2.4	58 ± 1.7
Skip	75 ± 2.0	100 ± 2.5	84 ± 2.2	69 ± 2.0	95 ± 2.4	72 ± 2.2
Ariel	65 ± 1.8	60 ± 1.7	61 ± 1.7	55 ± 1.6	51 ± 1.5	50 ± 1.5
OMO	100 ± 2.5	95 ± 2.4	70 ± 2.2	96 ± 2.4	88 ± 2.1	61 ± 1.7

Values represent the mean of three replicates and standard errors are reported.

Table 3. Stability of the purified SAPB and KERAB proteases in the presence of various commercial laundry detergents. The non-incubated enzyme was considered as 100%. The activity is expressed as a percentage of the activity level in the absence of organic solvent.

5.3 Wash performance analysis of SAPB

In order to evaluate the performance of SAPB in terms of ability to remove harsh stains, namely those caused by chocolate or human blood, several pieces of stained cotton cloth were incubated at different conditions (Fig. 3). The findings from these assays revealed that the blood and chocolate stain removal levels achieved with the use of SAPB alone were more effective than the ones obtained with detergent (Det) alone. In fact, SAPB facilitated the release of proteinacious materials in a much easier way than the commercialized SB 309 protease (Jaouadi et al., 2009). Furthermore, the combination of SAPB and the Det detergent resulted in complete stain removal (Fig. 3). In fact, a similar study has previously reported on the usefulness of alkaline proteases from *Spilosoma obliqua* (Anwar and Saleemuddin, 1997) and *B. brevis* (Banerjee et al., 1999) in the assistance of blood stain removal from cotton

cloth both in the presence and absence of detergents, but, in terms of reported results, the SAPB enzyme was more effective.

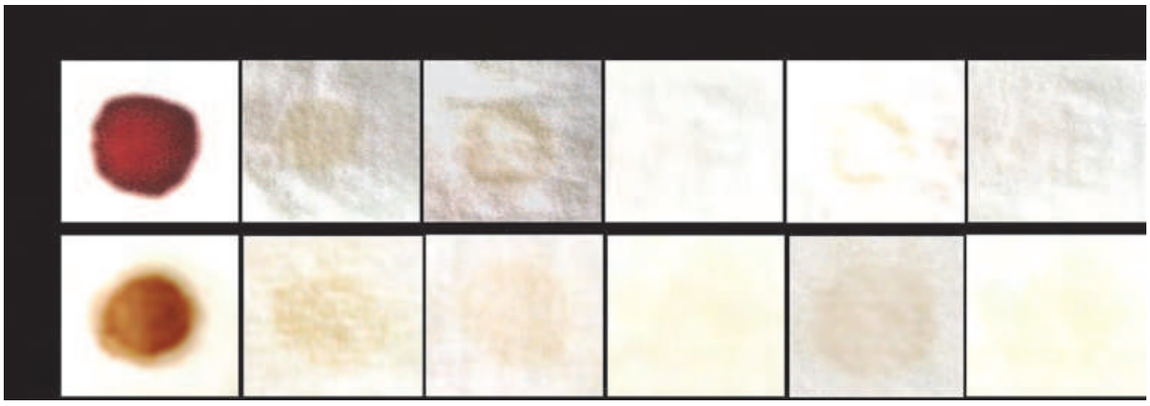


Fig. 3. Example of washing performance analysis test of SAPB. Stained cloth pieces with blood (I) or chocolate (II). (A) Control: untreated stained cloth pieces; or stained cloth pieces washed with: (B) distilled water, (C) Det detergent (7 mg/ml), (D) SAPB (500 U/ml), (E) SB 309 (commercial enzyme, 500 U/ml), and (F) SAPB (500 U/ml) + Det detergent (7 mg/ml).

5.4 Storage stability of the spray-dried and lyophilized SAPB

	Condition	t = 2 months	t = 12 months
		Residual activity (%)	
Spray-died	SAPB alone	76	55
	SAPB + Xylitol	88	70
	SAPB + Det	64	50
	SAPB + Det + Xylitol	78	70
Lyophilized	SAPB alone	74	55
	SAPB + Xylitol	80	68
	SAPB + Det	61	50
	SAPB + Det + Xylitol	75	65

Values are the means of three independent experiments.

Table 4. Stability of the spray-dried and lyophilized SAPB with and without xylitol at 1% during storage at room temperature and during prolonged storage within Det solid detergent. The activity of each treated SAPB before incubation was taken as 100% and the residual activity was determined at regular intervals.

The findings indicated that spray-dried SAPB, from fermentor culture, lost about 3% of its original activity; lyophilized SAPB lost about 10% (Jaouadi et al., 2009). Several of the additives used during the spray drying and lyophilizing processes were noted to improve SAPB stability (Table 4). However, the best results were actually obtained with 1% of xylitol, maltodextrin, and PEG 8000, which preserved about 100, 99 and 97% of its proteolytic activity, respectively. The stability of the spray-dried and lyophilized SAPB during subsequent storage in the presence of 1% xylitol showed that, after incubation at room

temperature for 12 months, the enzymes lost only about 20 and 25% of their original activity, respectively, against 35% for the control without additives. The non-treated enzyme was rapidly inactivated, losing about 50% of its initial activity after 2 months of incubation. Moreover, compared to the treated enzyme and in the absence of additives, 1% xylitol clearly enhanced SAPB stability during storage within the Det solid detergent (Jaouadi et al., 2009). In fact, after being incubated for 12 months at room temperature in the presence and absence of xylitol, the spray-dried enzyme retained 68 and 55% of its initial activity, respectively. The level of stability enhancement achieved for the lyophilized SAPB by xylitol was, on the other hand, less pronounced, since the enzyme retained only 65% of its initial activity.

6. Potential and prospects for SAPB and KERAB in the leather processing industry

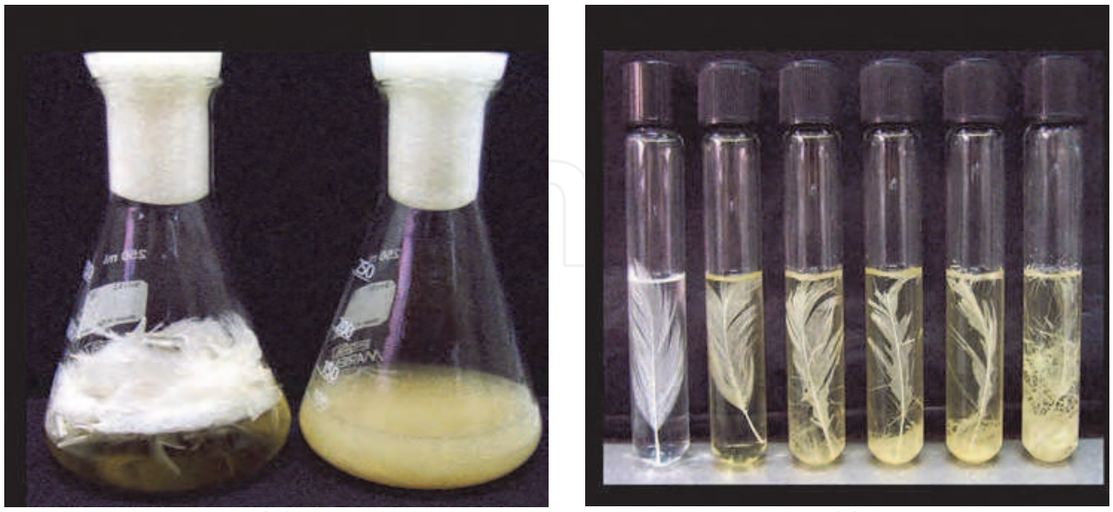
6.1 Keratin-degradation profile of *Bacillus pumilus* CBS and *Streptomyces* sp. AB1

Keratinacious substrates, such as keratin and keratin azure, were previously reported to be significantly hydrolyzed by SAPB (Jaouadi et al., 2008) and KERAB (Jaouadi et al., 2010a). It was also demonstrated that the *B. pumilus* strain CBS was able to grow in an optimized medium containing 10 g/l of feather-meal, chicken feather (Fig. 4A), goat hair, bovine hair, and sheep wool (as a sole carbon and nitrogen source) instead of gelatin and yeast extract, reaching an absorbance at 600 nm of 6 to 10 after 48 h-culture (Jaouadi et al., 2009). Of the 5 keratin substrates tested, feather-meal was the most strongly degraded (98.5%), followed by chicken feather (92%), goat hair (80%), and bovine hair (68%), with sheep wool showing a relatively low degradation rate (12%).

The feather-meal degradation rate achieved by *B. pumilus* CBS was higher than those of *B. pumilis* F3-4 (97%) (Son et al., 2008) and *Streptomyces albidoflavus* (67%) (Bressollier et al., 1999). The maximum release of protein obtained with the *B. pumilus* strain CBS occurred in the feather-meal medium, which was followed by the chicken feather medium. Moreover, while feather-meal and chicken feather gave the best SAPB production yields of 4,800 and 4,512 U/ml, respectively, sheep wool supported very low keratinolytic activity (1250 U/ml) (Jaouadi et al., 2009). Hence, its full-grown and intense Feather-Degrading (FD) activity could be achieved, in 24 h, at the range of 30 - 37ºC, and with initial pH adjusted from 8 to 9. This profile contrasts with previously reported results stipulating that *B. pumilus* FH9 solubilize feather in 72 h at 55°C with pH 9 (El-Refai et al., 2005) while *B. pumilis* F3-4 show intense FD activity in 168 h at 30°C with pH 7.5 (Son et al., 2008).

An increase simultaneous to keratin degradation was noted in protein levels and sulfhdryl groups (Jaouadi et al., 2009). Higher levels of keratin degradation resulted in high sulfhydryl group formation. The results obtained, therefore, suggested that *B. pumilus* CBS had a disulfide bond-reducing ability. Moreover, the processing of data from amino acid analysis following keratin degradation revealed a marked increase in the release of free amino acids after 12 h of incubation. The profile suggested that phenylalanine, tryptophan, leucine, isoleucine, valine, and alanine were the major amino acids liberated, whereas the untreated keratin (control) did not release any free amino acids. In fact, this amino acid profile matched well with the one described for the keratinolytic serine-enzyme produced by *B. licheniformis* PWD-1 (Williams et al., 1990). When SAPB was shaking-incubated with a white feather, a partial degradation was observed after 24 h with a simultaneous increase in protein concentration and sulfhydryl group formation, whereas no degradation was noticed

with the control (Fig. 4B). These results confirmed that SAPB alone could accomplish the whole dehairing process.



(A) (B)

Fig. 4. Keratin(feather)-degradation by *B. pumilus* strain CBS and SAPB. (A) Feathers were incubated for 24 h at 37°C under shake culture condition with 2.8×10^8 cells/ml as an initial inoculum density of the strain CBS (right flask) and with autoclaved inoculum as control (left flask). (B) SABP was incubated for 24 h at 37°C with chicken feather.

The *Streptomyces* sp. strain AB1 was able to grow after 2 days of culture in a mineral salt medium containing 30 g/l of intact chicken feathers as sole carbon, nitrogen, and sulfur sources instead of 10 g/l of feather meal. Intense feather-degrading activity was achieved at 30 °C and initial pH 9 (Jaouadi et al., 2010a). Interestingly, a nearly complete feather degradation was achieved, including the delamination of the rachis. A simultaneous increase of protein concentration and sulfhydryl group formation followed by a higher disulfide bond-reducing activity of KERAB were also observed. In contrast, no degradation was noted with the control. These results, therefore, suggested that the *Streptomyces* sp. strain AB1 had a disulfide bond-reducing ability. Furthermore, when KERAB was incubated with native chicken feathers, total degradation was observed after 24 h with a simultaneous increase of protein concentration and sulfhydryl group formation; whereas no degradation was noted with the control (Jaouadi et al., 2009).

The use of enzymatic and/or microbiological methods for the hydrolysis of feathers is an attractive alternative to the currently used methods of feather meal preparation which involve high temperature and pressure treatments that result in the loss of essential amino acids (Hess and FitzGerald, 2007). The ability of the *B. pumilus* strain CBS and *Streptomyces* sp. strain AB1 to grow and produce appreciable levels of protease and keratinase using feather as a substrate could open new opportunities for the achievement of efficient biodegradation and valorization processes of keratin-containing wastes and, thereby, help reduce the environmental impact of such biowaste.

6.2 Dehairing utility of SAPB

The incubation of the SAPB protease with skin from goat (Fig. 5), bovine (Jaouadi et al., 2009), and sheep (Jaouadi et al., 2009) for dehairing showed that after 24 h-incubation at

37°C, hair was removed very easily for all skins, compared to the corresponding controls, with no observable damage on the collagen. Therefore, the dehaired skins obtained exhibited clean hair pore and clear grain structure (data not shown). Again, these results confirmed that SAPB alone could accomplish the whole dehairing process.
The dehairing operation in leather processing is generally carried out under a relatively high pH value of about 8 - 10 (Dayanandan et al., 2003). This criterion was also satisfied by SAPB. In fact, approximately similar results were reached with the *A. tamarri* alkaline protease on goat skin at pH 9 - 11 and temperature of 30-37°C (Dayanandan et al., 2003). Likewise, similar results were obtained with the *Vibrio* sp. strain Kr2 but at pH values ranging from 6 to 8 and temperature of 30°C (Grazziotin et al., 2007). Other alkaline proteases from *B. pumilus* with high keratinolytic activity were also reported to accomplish alone the dehairing process on bovine hair (Kumar et al., 2008), cowhides (Wang et al., 2007a) and goatskins (Huang et al., 2003). However, with the higher dehairing ability and FD activities reported for SAPB, the latter could be considered a potential strong candidate for application in biotechnological bioprocesses involving the dehairing of hides or skins or the conversion of feather-rich wastes into economically useful feather-meal.

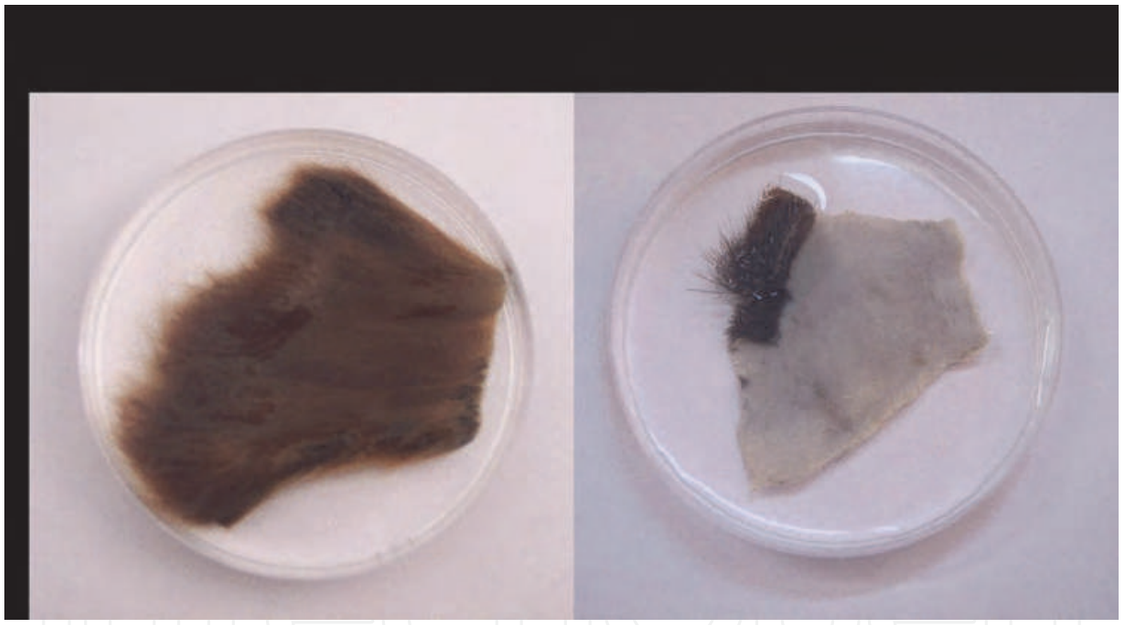


Fig. 5. Dehairing function of SAPB. SABP was incubated for 24 h at 37°C with, goat hair. (Left = control, Right = test).

7. Effect of organic solvents on the activity and stability of SAPB and KERAB

In addition to the key areas of application discussed for proteases above, the latter constitute a highly resourceful class of enzymes for various industrial sectors. They are, for instance, necessary in the biocatalysis of various peptide coupling reactions, which are of an extremely pharmaceutical and nutritional interest, namely those involved in the synthesis of several drug precursors such as the enkephalin (Kimura et al., 1990) and aspartame precursors (Nakanishi et al., 1990). However, the ultimate application of proteases in the synthesis of peptides has often been curtailed by the poor levels of specificity and instability

in the presence of organic solvents so far reported in the literature. Accordingly, various water-miscible organic solvents and alcohols at final concentrations of 50% were assayed for their effect on SAPB and KERAB activity at pH 10 and 60°C. Buthanol, acetonitrile, and ethyl acetate had significant inhibitory effects on the activity of both enzymes (Table 5). By contrast, dimethylformamide (DMF), DMSO, and hexane were noted to enhance the activity and stability of both enzymes while isopropanol and ethanol enhanced those of SAPB and KERAB, respectively (Table 5). Hence, good stability rates of 115, 97, 90 and 85% were exhibited by SAPB in the presence of DMF, hexane, isopropanol and DMSO, respectively. Equally good stability rates of 150, 125, 115 and 105% were displayed by KERAB in the presence DMSO, DMF, ethanol, and haxane, respectively. Acetonitrile, however, exerted a considerably negative effect on enzyme stability. Compared to SAPB, NH1 (Hadj-Ali et al., 2007) seemed less efficient for it exhibited only 181.5 and 94.5% of its initial activity and stability in the presence of 25% DMSO, respectively. The exception was observed with the organic solvent-tolerant protease BG1 (Ghorbel-Frikha et al., 2005; Ghorbel et al., 2003), which showed a half-life of 50 days of its activity in the presence of 25% DMSO. While the only report available to date on organic solvent protease from *B. pumilus* 115b (Rahman et al., 2007) showed that it exhibited 134% of its initial activity in the presence of 25% hexane as opposed to the 190% for SAPB and 145% for KERAB.

Organic solvent (50%)	**Relative activity (%)**		**Residual activity (%)**	
	SAPB	**KERAB**	**SAPB**	**KERAB**
None	100 ± 2.5	100 ± 2.5	100 ± 2.5	100 ± 2.5
Methanol	100 ± 2.1	80 ± 2.1	85 ± 2.2	75 ± 2.0
Ethanol	75 ± 2.0	132 ± 3.2	55 ± 2.6	115 ± 2.6
Buthanol	50 ± 1.4	79 ± 2.0	38 ± 1.4	63 ± 1.5
Isopropanol	115 ± 2.6	25 ± 0.5	90 ± 2.3	15 ± 0.8
Acetonitrile	25 ± 1.0	20 ± 1.0	10 ± 0.8	0 ± 0.1
Ethyl acetate	85 ± 2.2	66 ± 1.5	72 ± 2.0	58 ± 1.5
DMF	200 ± 5.0	155 ± 3.7	115 ± 3.0	125 ± 3.0
DMSO	150 ± 3.7	195 ± 4.9	85 ± 2.2	150 ± 3.7
Hexane	170 ± 4.0	160 ± 3.8	97 ± 2.5	105 ± 2.5

Values represent the mean of three replicates and standard errors are reported.

Table 5. Effect organic solvents on SAPB and KERAB activity and stability. The non-incubated enzyme was considered as 100%. The activity is expressed as a percentage of the activity level in the absence of organic solvent.

A combination of high esterase and low amidase activities is necessary for several synthetic applications of proteases, including peptides coupling (Plettner et al., 1999). In addition to demonstrating its organic tolerance, the findings presented above show that both SAPB and KERAB exhibited powerful esterase activities on BTEE and on BAEE. Furthermore, no amidase activity was detected for SAPB and KERAB on BAEE with P_1= Arg and ATEE with P1 = Tyr, respectively. These findings, in addition of the observed activity and stability in certain organic solvents strongly suggested that SAPB and KERAB are potential strong candidates for use in peptide synthesis reactions in low water systems.

8. Conclusion

This chapter described the valuable advantages inherent in proteases and the promising opportunities they offer for the enhancement of a variety of industrial and consumer product applications. This was illustrated by an overview on the purification and characterization of two extracellular extremozyme serine alkaline proteinases, namely SAPB and KERAB, which were isolated from *B. pumilus* strain CBS and *Streptomyces* sp. strain AB1, respectively. These pure enzymes were significantly tolerant and stable in the presence of the various laundry detergents tested, which strongly supported their suitablity for liquid and solid laundry detergents. Furthermore, and in comparison with the standard enzyme, namely SB 309, both SAPB and KERAB turned to be more effective under alkaline and high temperature conditions. Furthermore, the *B. pumilus* strain CBS and *Streptomyces* sp. strain AB1 proved suitable for the degradation of avian feathers and feather-meal, showing strong potential for application in future biotechnological processes. More interestingly, SAPB demonstrated powerful dehairing abilities against various skins with minimal damage on collagen. Last but not least, these enzymes showed high esterase and low amidase activities as well a good tolerance for several organic solvents. Overall, the findings presented in this chapter strongly suggest that both enzymes, SAPB and KERAB, offer new and promising opportunities for prospective application in biotechnological bioprocesses, particularly those involving the synthesis of detergent formulations, dehairing during leather processing, and peptide biocatalysis in non-aqueous environments.

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10. References

Anonyme (2007). World enzymes to 2011 (2229). *Focus on Catalysts* 2007, 2-2.

Anwar, A. & Saleemuddin, M. (1997). Alkaline-pH-acting digestive enzymes of *Polyphagous brevis* and its characterization as a laundry detergent additive. *Process Biochem*. 35, 213-216.

Awad, W. M., Jr., Soto, A. R., Siegel, S., Skiba, W. E., Bernstrom, G. G. & Ochoa, M. S. (1972). The proteolytic enzymes of the K-1 strain of *Streptomyces griseus* obtained from a commercial preparation (Pronase). I. Purification of four serine endopeptidases. *J Biol Chem*. 247, 4144-4154.

Badis, A., Ferradji, F. Z., Boucherit, A., Fodil, D. & Boutoumi, H. (2010). Characterization and biodegradation of soil humic acids and preliminary identification of decolorizing actinomycetes at Mitidja plain soils (Algeria). *Afr J Microbiol Res*. 3, 997-1007.

Banerjee, U., Sani, R., Azmi, W. & Sani, R. K. (1999). Thermostable alkaline protease from *Bacillus brevis* and its characterisation as a laundry detergent additive. *Process Biochem*. 35, 213-219.

Beg, Q. & Gupta, R. (2003). Purification and characterization of an oxidation stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microb Technol*. 32, 294-304.

Bertsch, A. & Coello, N. (2005). A biotechnological process for treatment and recycling poultry feathers as a feed ingredient. *Bioresour Technol*. 96, 1703-1708.

Brandelli, A., Daroit, D. J. & Riffel, A. (2010). Biochemical features of microbial keratinases and their production and applications. *Appl Microbiol Biotechnol*. 85, 1735-1750.

Bressollier, P., Letourneau, F., Urdaci, M. & Verneuil, B. (1999). Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. *Appl Environ Microbiol*. 65, 2570-2576.

Chitte, R. R., Nalawade, V. K. & Dey, S. (1999). Keratinolytic activity from the broth of a feather-degrading thermophilic *Streptomyces thermoviolaceus* strain SD8. *Lett Appl Microbiol*. 28, 131-136.

Coulombe, P. A. & Omary, M. B. (2002). 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr Opin Cell Biol*. 14, 110-122.

Dayanandan, A., Kanagaraj, J., Sounderraj, L., Govindaraju, R. & Rajkumar, G. S. (2003). Application of an alkaline protease in leather processing: An ecofriendly approach. *Journal Clean Prod*. 11, 533-536.

DelMar, E. G., Largman, C., Brodrick, J. W. & Geokas, M. C. (1979). A sensitive new substrate for chymotrypsin. *Anal Biochem*. 99, 316-320.

El-Refai, H. A., AbdelNaby, M. A., Gaballa, A., El-Araby, M. H. & Abdel Fattah, A. F. (2005). Improvement of the newly isolated *Bacillus pumilus* FH9 keratinolytic activity. *Process Biochem*. 40, 2325-2332.

Essien, J. P., Umoh, A. A., Akpan, E. J., Eduok, S. I. & Umoiyoho, A. (2009). Growth, keratinolytic proteinase activity and thermotolerance of dermatophytes associated with alopecia in Uyo, Nigeria. *Acta Microbiol Immunol Hung*. 56, 61-69.

Fakhfakh-Zouari, N., Hmidet, N., Haddar, A., Kanoun, S. & Nasri, M. (2010). A novel serine metallokeratinase from a newly isolated *Bacillus pumilus* A1 grown on chicken feather meal: biochemical and molecular characterization. *Appl Biochem Biotechnol*. 162, 329-344.

Ghorbel-Frikha, B., Sellami-Kamoun, A., Fakhfakh, N., Haddar, A., Manni, L. & Nasri, M. (2005). Production and purification of a calcium-dependent protease from *Bacillus cereus* BG1. *J Ind Microbiol Biotechnol*. 32, 186-194.

Ghorbel, B., Sellami-Kamoun, A. & Nasri, M. (2003). Stability studies of protease from *Bacillus cereus* BG1. *Enzyme Microb Technol*. 32, 513-518.

Grazziotin, A., Pimentel, F. A., Sangali, S., de Jong, E. V. & Brandelli, A. (2007). Production of feather protein hydrolysate by keratinolytic bacterium *Vibrio* sp. kr2. *Bioresour Technol*. 98, 3172-3175.

Gupta, R., Beg, Q. K. & Lorenz, P. (2002). Bacterial alkaline proteases: Molecular approaches and industrial applications. *Appl Microbiol Biotechnol*. 59, 15-32.

Gupta, R., Gupta, K., Saxena, R. & Khan, S. (1999). Bleach-stable alkaline protease from & sp. *Biotechnol Lett*. 21, 135-138.

Gupta, R., Saxena, R. K., Chaturvedi, P. & Virdi, J. S. (1995). Chitinase production by *Streptomyces viridificans*: Its potential in fungal cell wall lysis. *J Appl Bacteriol*. 78, 378-383.

Haddar, A., Agrebi, R., Bougatef, A., Hmidet, N., Sellami-Kamoun, A. & Nasri, M. (2009). Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: Purification, characterization and potential application as a laundry detergent additive. *Bioresour Technolo*.100, 3366-3373.

Hadj-Ali, N. E., Agrebi, R., Ghorbel-Frikha, B., Sellami-Kamoun, A., Kanoun, S. & Nasri, M. (2007). Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1. *Enzyme Microb Technol*. 40, 515-523.

Han, X.-Q. & Damodaran, S. (1998). Purification and Characterization of Protease Q: A detergent- and urea-stable serine endopeptidase from *Bacillus pumilus*. *J Agric Food Chem*. 46, 3596-3603.

Hess, J. F. & FitzGerald, P. G. (2007). Treatment of keratin intermediate filaments with sulfur mustard analogs. *Biochem Biophys Res Commun*. 359, 616-621.

Huang, Q., Peng, Y., Li, X., Wang, H. & Zhang, Y. (2003). Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. *Curr Microbiol*. 46, 169-173.

Ignatova, Z., Gousterova, A., Spassov, G. & Nedkov, P. (1999). Isolation and partial characterisation of extracellular keratinase from a wool degrading thermophilic actinomycete strain *Thermoactinomyces candidus*. *Can J Microbiol*. 45, 217-222.

Jaouadi, B., Abdelmalek, B., Fodil, D., Ferradji, F. Z., Rekik, H., Zaraî, N. & Bejar, S. (2010a). Purification and characterization of a thermostable keratinolytic serine alkaline proteinase from *Streptomyces* sp. strain AB1 with high stability in organic solvents. *Bioresour Technol*. 101, 8361-8369.

Jaouadi, B., Aghajari, N., Haser, R. & Bejar, S. (2010b). Enhancement of the thermostability and the catalytic efficiency of *Bacillus pumilus* CBS protease by site-directed mutagenesis. *Biochimie*. 92, 360-369.

Jaouadi, B. & Bejar, S. (2008). Characterization of an original serine alkaline proteinase from *Bacillus pumilus* CBS. *J Biotechnol*.136 (Suppl.) S305.

Jaouadi, B., Ellouz-Chaabouni, S., Ben Ali, M., Ben Messaoud, E., Naili, B., Dhouib, A. & Bejar, S. (2009). Excellent laundry detergent compatibility and high dehairing ability of the *Bacillus pumilus* CBS alkaline proteinase (SAPB). *Biotechnol Bioprocess Eng*. 14, 503-512.

Jaouadi, B., Ellouz-Chaabouni, S., Rhimi, M. & Bejar, S. (2008). Biochemical and molecular characterization of a detergent-stable serine alkaline protease from *Bacillus pumilus* CBS with high catalytic efficiency. *Biochimie*. 90, 1291-305.

Johnson, P. & Smillie, L. B. (1974). The amino acid sequence and predicted structure of *Streptomyces griseus* protease A. *FEBS Lett*. 47, 1-6.

Johnvesly, B. & Naik, G. (2001). Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. *Process Biochem*. 37, 139-144.

Kimura, Y., Nakanishi, K. & Matsuno, R. (1990). Enzymatic synthesis of the precursor of Leu-enkephalin in water-immiscible organic solvent systems. *Enzyme Microb Technol*. 12, 272-280.

Kirk, O., Borchert, T. V. & Fuglsang, C. C. (2002). Industrial enzyme applications. *Curr Opin Biotechnol*. 13, 345-351.

Kitadokoro, K., Tsuzuki, H., Nakamura, E., Sato, T. & Teraoka, H. (1994). Purification, characterization, primary structure, crystallization and preliminary crystallographic study of a serine proteinase from *Streptomyces fradiae* ATCC 14544. *Eur J Biochem*. 220, 55-61.

Kumar, A. G., Swarnalatha, S., Gayathri, S., Nagesh, N. & Sekaran, G. (2008). Characterization of an alkaline active-thiol forming extracellular serine keratinase by the newly isolated *Bacillus pumilus*. *J Appl Microbiol*. 104, 411-420.

Kumar, C. G. (2002). Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. *Lett Appl Microbiol*. 34, 13-17.

Maurer, K. H. (2004). Detergent proteases. *Curr Opin Biotechnol*. 15, 330-334.

Miyaji, T., Otta, Y., Nakagawa, T., Watanabe, T., Niimura, Y. & Tomizuka, N. (2006). Purification and molecular characterization of subtilisin-like alkaline protease BPP-A from *Bacillus pumilus* strain MS-1. *Lett Appl Microbiol*. 42, 242-247.

Nakanishi, K., Takeuchi, A. & Matsuno, R. (1990). Long-term continuous synthesis of aspartame precursor in a column reactor with an immobilized thermolysin. *Appl Microbiol Biotechnol*. 32, 633-636.

Onifade, A. A., Al-Sane, N. A., Al-Musallam, A. A. & Al-Zarban, S. (1998). A review: Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. *Bioresour Technol*. 66, 1-11.

Pillai, P. & Archana, G. (2008). Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel *Bacillus subtilis* isolate. *Appl Microbiol Biotechnol*. 78, 643-650.

Plettner, E., DeSantis, G., R. Stabile, M. & Jones, J. B. (1999). Modulation of esterase and amidase activity of subtilisin bacillus lentus by chemical modification of cysteine mutants. *J Am Chem Soc*. 121 4977-4981.

Radha, S. & Gunasekaran, P. (2008). Sustained expression of keratinase gene under PxylA and PamyL promoters in the recombinant *Bacillus megaterium* MS941. *Bioresour Technol*. 99, 5528-5537.

Rahman, R. N., Mahamad, S., Salleh, A. B. & Basri, M. (2007). A new organic solvent tolerant protease from *Bacillus pumilus* 115b. *J Ind Microbiol Biotechnol*. 34, 509-517.

Rahman, R. N. Z. A., Razak, C. N., Ampon, K., Basri, M., Zin, W. M., Yunus, W. & Salleh, A. B. (1994). Purification and characterization of a heat-stable alkaline protease from *Bacillus stearothermophilus* F1. *Appl Microb Biotechnol*. 40, 822-827.

Rao, M. B., Tanksale, A. M., Ghatge, M. S. & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev*. 62, 597-635.

Rawlings, D. N. & Barrett, A. J. (1994). Families of serine peptidases. Methods Enzymol. 244, 19-61.

Rick, W. Trypsin. In: H.U. Bergmeyer, Editor, (2nd English edition.), Methods of Enzymatic Analysis. Vol. 2, Verlag Chemie, Weinheim (1974), pp. 1013-1024 Academic Press, New York, NY USA.

Riffel, A. & Brandelli, A. (2002). Isolation and characterization of a feather-degrading bacterium from the poultry processing industry. *J Ind Microbiol Biotechnol*. 29, 255-258.

Schoellman, G. & Shaw, E. (1963). Direct evidence for the presence of histidine in the active center of chympotrypsin. *Biochemistry*. 2, 252-255.

Singh, J., Batra, N. & Sobti, R. C. (2001). Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. *Process Biochem*. 36, 781-785.

Son, H. J., Park, H. C., Kim, H. S. & Lee, C. Y. (2008). Nutritional regulation of keratinolytic activity in *Bacillus pumilis*. *Biotechnol Lett*. 30, 461-465.

Syed, D. G., Lee, J. C., Li, W. J., Kim, C. J. & Agasar, D. (2009). Production, characterization and application of keratinase from *Streptomyces gulbargensis*. *Bioresour Technol*. 100, 1868-1871.

Szabo, I., Benedek, A. & Mihaly Szabo, I. B., G.Y. (2000). Feather degradation with a thermotolerant *Streptomyces graminofaciens* strain. *World J Microbiol Biotechnol*. 16, 153-255.

Tatineni, R., Doddapaneni, K. K., Potumarthi, R. C., Vellanki, R. N., Kandathil, M. T., Kolli, N. & Mangamoori, L. N. (2008). Purification and characterization of an alkaline keratinase from *Streptomyces* sp. *Bioresour Technol*. 99, 1596-1602.

Venugopal, M. & Saramma, A. V. (2006). Characterization of alkaline protease from *Vibrio fluvialis* strain VM10 isolated from a mangrove sediment sample and its application as a laundry detergent additive. *Process Biochem*. 41, 1239-1243.

Walsh, K. A. (1970). Trypsinogens and trypsins of varoius species. *Methods Enzymol*. 19, 41-63.

Wang, H. Y., Liu, D. M., Liu, Y., Cheng, C. F., Ma, Q. Y., Huang, Q. & Zhang, Y. Z. (2007a). Screening and mutagenesis of a novel *Bacillus pumilus* strain producing alkaline protease for dehairing. *Lett Appl Microbiol*. 44, 1-6.

Wang, S. L., Chio, Y. H., Yen, Y. H. & Wang, C. L. (2007b). Two novel surfactant-stable alkaline proteases from *Vibrio fluvialis* TKU005 and their applications. *Enzyme Microb Technol*. 40, 1213-1220.

Wang, S. L., Hsu, W. T., Liang, T. W., Yen, Y. H. & Wang, C. L. (2008). Purification and characterization of three novel keratinolytic metalloproteases produced by *Chryseobacterium indologenes* TKU014 in a shrimp shell powder medium. *Bioresour Technol*. 99, 5679-5686.

Williams, C. M., Richter, C. S., Mackenzie, J. M. & Shih, J. C. (1990). Isolation, identification, and characterization of a feather-degrading bacterium. *Appl Environ Microbiol*. 56, 1509-1515.

Xie, F., Chao, Y., Yang, X., Yang, J., Xue, Z., Luo, Y. & Qian, S. (2010). Purification and characterization of four keratinases produced by *Streptomyces* sp. strain 16 in native human foot skin medium. *Bioresour Technol*.101, 344-350.

Yasuda, M., Aoyama, M., Sakaguchi, M., Nakachi, K. & Kobamoto, N. (1999). Purification and characterization of a soybean-milk-coagulating enzyme from *Bacillus pumilus* TYO-67. *Appl Microbiol Biotechnol*. 51, 474-479.

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