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## Bacterial alkaline proteases: molecular approaches and industrial applications

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**Abstract** Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers. A good number of bacterial alkaline proteases are commercially available, such as subtilisin Carlsberg, subtilisin BPN' and Savinase, with their major application as detergent enzymes. However, mutations have led to newer protease preparations with improved catalytic efficiency and better stability towards temperature, oxidizing agents and changing wash conditions. Many newer preparations, such as Durazym, Maxapem and Purafect, have been produced, using techniques of site-directed mutagenesis and/or random mutagenesis. Directed evolution has also paved the way to a great variety of subtilisin variants with better specificities and stability. Molecular imprinting through conditional lyophilization is coming up to match molecular approaches in protein engineering. There are many possibilities for modifying biocatalysts through molecular approaches. However, the search for microbial sources of novel alkaline proteases in natural diversity through the "metagenome" approach is targeting a hitherto undiscovered wealth of molecular diversity. This fascinating development will allow the biotechnological exploitation of uncultured microorganisms, which by far outnumber the species accessible by cultivation, regardless of the habitat. In this review, we discuss the types and sources of proteases, protease yield-improvement methods, the use of new methods for developing novel proteases and applications of alkaline

proteases in industrial sectors, with an overview on the use of alkaline proteases in the detergent industry.

### Introduction

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community. These enzymes have become widely used in the detergent industry, since their introduction in 1914 as detergent additives. Microorganisms elaborate a large array of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products (Kalisz 1988). At the same time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes (Kumar and Takagi 1999; Outtrup and Boyce 1990).

Today, proteases account for approximately 40% of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery. This dominance of proteases in the industrial market is expected to increase further by the year 2005 (Godfrey and West 1996). However, until today, the largest share of the enzyme market has been held by detergent alkaline proteases active and stable in the alkaline pH range. Microbial proteases have been reviewed several times, with emphasis on different aspects of proteases. Aunstrup (1980) focused on microbial selection and fermentation of proteases, whereas Ward (1985) mainly dealt with the

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sources of microbial proteases and their possible functional role in nature. Kalisz (1988) updated the available information with a detailed description of the types of proteases and their commercial applications, whereas Outtrup and Boyce (1990) focused on the industrially important proteases, their applications and the role of molecular biology in protease research. Rao et al. (1998) published an excellent review, in terms of molecular biology, biochemical and genetic aspects of microbial, animal and plant proteases. The bioindustrial viewpoints of microbial alkaline proteases from sources to cellular role, production, downstream processing, characterization and commercial applications have also been reviewed (Anwar and Saleemuddin 1998; Kumar and Takagi 1999). In this present review, some aspects of microbial alkaline proteolytic enzymes are discussed, with reference to their industrial applications and an emphasis on the detergent industry, along with the upcoming approaches for discovering and developing novel proteases using new technologies. The major focus in this review has been kept on serine alkaline proteases from bacteria.

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## Microbial proteolytic system

Proteases are essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals. They can be cultured in large quantities in a relatively short time by established methods of fermentation and they also produce an abundant, regular supply of the desired product. Microorganisms account for a two-third share of commercial protease production in the world (Kumar and Takagi 1999). Microbial proteases are classified into various groups, dependent on whether they are active under acidic, neutral, or alkaline conditions and on the characteristics of the active site group of the enzyme, i.e. metallo- (EC.3.4.24), aspartic- (EC.3.4.23), cysteine- or sulphhydryl- (EC.3.4.22), or serine-type (EC.3.4.21; Kalisz 1988; Rao et al. 1998). Alkaline proteases (EC.3.4.21–24, 99) are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine center (serine protease) or are of metallo-type (metalloprotease); and the alkaline serine proteases are the most important group of enzymes exploited commercially.

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## Types of serine proteases

Serine proteases are of considerable interest, in view of their activity and stability at alkaline pH; and thus they have applications in a number of industries. They have a nucleophilic serine residue located in their active site. Apart from this, these proteases are also distinguished by having essential aspartate and histidine residues which, along with the serine, forms the catalytic triad. They are generally active at neutral and alkaline pH, with optima at pH 7–11, although higher pH optima (10.0–12.5) from *Bacillus* sp. YaB have also been reported (Shimogaki et

al. 1991). They show broad substrate specificity and, with few exceptions [like a serine protease of high molecular mass (90 kDa) from *B. subtilis* (*natto*) (Kato et al. 1992; Yamagata et al. 1995a)], they are generally of low molecular mass (18–35 kDa). The serine proteases are further classified on the basis of their side-chain specificity against the oxidized insulin  $\beta$ -chain (Moriyama 1974) and structural homology to well established proteases. Various types of serine proteases have been described in great detail in the earlier reviews (Kalisz 1988; Moriyama 1974; Rawlings et al. 1991; Siezen and Leunissen 1997). A comprehensive account of subclasses of serine proteases is presented below for ready reference.

### Chymotrypsin-like proteases

Chymotrypsin, a mammalian digestive protease, has structural homology with trypsin, elastase and thrombin (Graycar 1999). It is specific for basic amino acids and is most active at pH 8. The most important inhibitors of this class are *N*-tosyl-L-lysine chloromethyl ketone, L-1-tosylamide-2-phenylethyl chloromethyl ketone, diisopropylfluorophosphate and soyabean trypsin inhibitor. The molecular weight is generally around 20 kDa. This group is well represented by proteases of animal origin and those belonging to various species of *Streptomyces*, like *S. erythreus*, *S. fradiae* and *S. griseus* (Ward 1985; Kalisz 1988).

### Subtilisin-like proteases or subtilases

Subtilisin-like serine proteases are generally bacterial in origin, although there are reports in favor of other organisms also (Siezen and Leunissen 1997). They are generally secreted extracellularly for the purpose of scavenging nutrients (Graycar 1999). This class of proteases is specific for aromatic or hydrophobic residues (at position P1), such as tyrosine, phenylalanine and leucine. They are highly sensitive towards phenyl methyl sulphonyl fluoride, diisopropyl-fluorophosphate and potato inhibitor. They are most active around pH 10, with a molecular weight range of 15–30 kDa and an isoelectric point near pI 9. This class of protease is well represented by various species of *Bacillus*, like *B. amyloliquifaciens*, *B. licheniformis* and *B. subtilis* (Rao et al. 1998). It is also produced by *Flavobacterium* (Morita et al. 1998).

### Wheat serine carboxypeptidase II-like proteases

These have a different catalytic triad residue in their amino acid sequence and their tertiary folds are also completely different for each structural family. They are most active in the pH range 4.5–5.5, suggesting an unusual low  $pK_a$  value for its catalytic histidine residue (Liao and Remington 1990).

### Prolyl oligopeptidase-like serine proteases

Not much is known about this class of proteases but this group was defined based on the results obtained using sequence homology techniques (Rawlings et al. 1991).

### Myxobacter $\alpha$ -lytic proteases

These exhibit strong bacteriolytic activity towards soil bacteria and are represented by various species of *Sorangium*. They are specific towards the carboxyl group of neutral and aliphatic amino acids. They show maximum activity around pH 9 and are sensitive towards diisopropylfluorophosphate (Moriyama 1974).

### Staphylococcal protease

*Staphylococcus aureus* produces a diisopropylfluorophosphate-sensitive protease of 12 kDa with maximum

activity in the pH range 4.0–7.8. The enzyme is specific towards peptide bonds with acidic amino acid residues at the carboxylic group (Moriyama 1974).

## Bacterial sources and sequence homologies of alkaline proteases

Protease production is an inherent capacity of all microorganisms; and a large number of bacterial species are known to produce alkaline proteases of the serine-type, although very few are recognized as commercial producers. Only those microbes that produce substantial amounts of extracellular enzyme are of industrial importance. Several products based on bacterial alkaline proteases have been launched successfully in the market in past few years (Table 1).

Subtilisins are a prototypical group of bacterial serine proteases. The first report on DNA sequence determination was on Subtilisin BPN' from *B. amyloliquefaciens* (Wells et al. 1983). Since then, nucleotide sequences of

**Table 1** Commercial bacterial alkaline proteases, sources, applications and their industrial suppliers. *n.s.* Not specified

Supplier	Product trade name	Microbial source	Application
Novo Nordisk, Denmark	Alcalase	<i>Bacillus licheniformis</i>	Detergent, silk degumming
	Savinase	<i>Bacillus</i> sp.	Detergent, textile
	Esperase	<i>B. lentus</i>	Detergent, food, silk degumming
	Biofeed pro	<i>B. licheniformis</i>	Feed
	Durazym	<i>Bacillus</i> sp.	Detergent
	Novozyme 471MP	<i>n.s.</i>	Photographic gelatin hydrolysis
	Novozyme 243	<i>B. licheniformis</i>	Denture cleaners
	Nue	<i>Bacillus</i> sp.	Leather
Genencor International, USA	Purafact	<i>B. lentus</i>	Detergent
	Primatan	Bacterial source	Leather
Gist-Brocades, The Netherlands	Subtilisin	<i>B. alcalophilus</i>	Detergent
	Maxacal	<i>Bacillus</i> sp.	Detergent
	Maxatase	<i>Bacillus</i> sp.	Detergent
Solvay Enzymes, Germany	Opticlean	<i>B. alcalophilus</i>	Detergent
	Optimase	<i>B. licheniformis</i>	Detergent
	Maxapem	Protein engineered variant of <i>Bacillus</i> sp.	Detergent
	HT-proteolytic	<i>B. subtilis</i>	Alcohol, baking, brewing, feed, food, leather, photographic waste
Amano Pharmaceuticals, Japan	Protease	<i>B. licheniformis</i>	Food, waste
	Proleather	<i>Bacillus</i> sp.	Food
	Collagenase	<i>Clostridium</i> sp.	Technical
	Amano protease S	<i>Bacillus</i> sp.	Food
Enzyme Development, USA	Enzeco alkaline protease	<i>B. licheniformis</i>	Industrial
	Enzeco alkaline protease-L FG	<i>B. licheniformis</i>	Food
	Enzeco high alkaline protease	<i>Bacillus</i> sp.	Industrial
Nagase Biochemicals, Japan	Biopraser concentrate	<i>B. subtilis</i>	Cosmetic, pharmaceuticals
	Ps. protease	<i>Pseudomonas aeruginosa</i>	Research
	Ps. elastase	<i>Pseudomonas aeruginosa</i>	Research
	Cryst. protease	<i>B. subtilis</i> (K2)	Research
	Cryst. protease	<i>B. subtilis</i> (bioteus)	Research
	Biopraser	<i>B. subtilis</i>	Detergent, cleaning
	Biopraser SP-10	<i>B. subtilis</i>	Food
Godo Shusei, Japan	Godo-Bap	<i>B. licheniformis</i>	Detergent, food
Rohm, Germany	Corolase 7089	<i>B. subtilis</i>	Food
Wuxi Synder Bioproducts, China	Wuxi	<i>Bacillus</i> sp.	Detergent
Advance Biochemicals, India	Protosol	<i>Bacillus</i> sp.	Detergent



the subtilisins and other alkaline proteases, such as subtilisin E from *B. subtilis* (Stahl and Ferrari 1984), subtilisin Carlsberg from *B. licheniformis* (Jacobs et al. 1985), subtilisin amylosacchariticus from *B. subtilis* var. *amylosacchariticus* (Yoshimoto et al. 1988), subtilisin NAT from *B. subtilis* (*natto*) (Nakamura et al. 1992; Yamagata et al. 1995a) and alkaline proteases from *B. alcalophilus* PB92 (Van der Laan et al. 1991) and *Bacillus* sp. 221 (Takami et al. 1992c), have been determined. These studies on DNA and protein sequence similarity are necessary for a variety of purposes and have therefore become routine in computational molecular biology (Rao et al. 1998).

Knowledge of full nucleotide sequences of the enzyme genes has facilitated the deduction of the primary structure of the encoded enzymes and, in many cases, identification of various functional regions. These sequences also serve as the basis for phylogenetic analysis of proteins and assist in predicting the secondary structure of proteins, thereby helping in the study of structure–function relationships of the enzyme. An 80% similarity was found between the DNA sequence of the subtilisin structural genes from *B. subtilis* and *B. amyloliquefaciens*; and the translated mature coding sequence was 85% identical to the protein sequence of subtilisin BPN' (Stahl and Ferrari 1984). Nakamura et al. (1992) reported that the nucleotide sequence of the subtilisin NAT gene from *B. subtilis* (*natto*) was nearly identical to that of subtilisin E and subtilisin amylosacchariticus, with discrepancies only at 13 and 27 nucleotides, respectively, of the 1,473 nucleotides sequenced. The primary structure of the mature region of the NAT gene has 99.5% and 99.3% similarity with that of the primary structure of mature subtilisin E and subtilisin amylosacchariticus. The homology of the total amino acid residues between subtilisin NAT and subtilisin BPN' or subtilisin Carlsberg was 86% or 72%, respectively; and sequences were conserved around three essential amino acids (Ser221, His64, Asp32) in the catalytic center (Nakamura et al. 1992). This sequence around the catalytic triad of these three amino acids is highly conserved among alkaline proteases and it has been suggested that the gene for both the intracellular and extracellular proteases evolved from a common ancestor by divergent evolution (Neurath 1984). Similarly, Koide et al. (1986) compared the amino acid sequences of intracellular serine protease from *B. subtilis* with those of subtilisin BPN' and subtilisin Carlsberg and showed 45% identity between them. Figure 1 shows a sequence alignment of some of the alkaline proteases deposited in GenBank. The alignment was created using CLUSTAL W and the

BLOSUM 30 Matrix in a Macintosh software package called MacVector.

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## Protease fermentation and yield improvement

The overall cost of enzyme production and downstream processing is the major obstacle against the successful application of any technology in the enzyme industry. Researchers and process engineers have used several methods to increase the yields of alkaline proteases with respect to their industrial requirements. Recent approaches for increasing protease yield include screening for hyper-producing strains, cloning and over-expression, fed-batch, chemostat fermentations, and optimization of the fermentation medium through a statistical approach, such as response surface methodology.

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## Fermentation methods

Generally, proteases produced from microorganisms are constitutive or partially inducible in nature and, under most culture conditions, *Bacillus* species produce extracellular proteases during post-exponential and stationary phases. Extracellular protease production in microorganisms is also strongly influenced by media components, e.g. variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose (Beg et al. 2002a), and metal ions (Varela et al. 1996). Protease synthesis is also affected by rapidly metabolizable nitrogen sources, such as amino acids in the medium. Besides these, several other physical factors, such as aeration, inoculum density, pH, temperature and incubation, also affect the amount of protease produced (Hameed et al. 1999; Puri et al. 2002). In order to scale up protease production from microorganisms at the industrial level, biochemical and process engineers use several strategies to obtain high yields of protease in a fermentor. Controlled batch and fed-batch fermentations using simultaneous control of glucose, ammonium ion concentration, oxygen tension, pH and salt availability (Hameed et al. 1999; Hubner et al. 1993; Mao et al. 1992; Van Putten et al. 1996) and chemostat cultures (Frankena et al. 1985, 1986) have been successfully used for improving protease production for long-term incubations, using a number of microorganisms. In a recent study by our group, the overall alkaline protease yield from *B. mojavensis* (Beg et al. 2002a) was improved up to 4-fold under semi-batch and fed-batch operations by separating biomass and protease production phases, using intermittent de-repression and induction during the growth of the organism. In recent years, there has been a great amount of research and development effort focusing on the use of statistical approach methods, using different statistical software packages (Table 2) during process optimization studies, with the aim of obtaining high yields of alkaline protease in the fermentation medium (De Coninck et al. 2000; Puri et al. 2002; Varela et al. 1996). The applica-

◀ **Fig. 1** Sequence alignment of alkaline proteases. The protease sequences were selected from the GenBank database and aligned using CLUSTAL W and the BLOSUM 30 matrix in a Macintosh software package called MacVector. The conserved residues are in dark boxes, while partial identity is shown by partially shaded boxes. Residues involved in the catalytic triad are marked with an asterisk

**Table 2** Statistical methods used to improve protease production from microorganisms. n.s. Not specified

Microorganism	Design	Software	Yield improvement	Reference
<i>Rhizopus oryzae</i>	Levenberg–Marquardt technique	n.s.	2.5-fold	Banerjee and Bhattacharyya (1992)
<i>Bacillus subtilis</i> HQDB32	Plackett–Burman	n.s.	3.0-fold	Varela et al. (1996)
<i>Tetrahymena thermophila</i> BIII	Central composite design	Minitab	1.2-fold (protease) 4.6-fold (biomass)	De Coninck et al. (2000)
<i>Mucor miehei</i> ATCC 3420 (acid protease)	Box–Wilson	n.s.	n.s.	Ayhan et al. (2001)
<i>Streptomyces</i> sp. 82 (elastase)	Box–Wilson	n.s.	7.5-fold	Georeva and Vlahov (2001)
<i>Bacillus</i> sp. RGR-14	Face-centered central composite design	Design-Expert (Statease)	2.6-fold	Puri et al. (2002)

tion of properly designed approaches with multi-factor models allows process and biochemical engineers to design scale-up strategies for increasing enzyme production.

Some other methods have been used for improving protease production from different microorganisms, such as cell immobilization of *B. firmus* 44 (Landau et al. 1995, 1997), aqueous two-phase (ATPase) systems composed of polyethylene glycol (PEG)-(4000, 6000, 9000) and potassium phosphate using *B. thuringiensis* (Hotha and Banik 1997) and the ATPase system composed of PEG-6000 and dextran T500 using *B. licheniformis* (Lee and Chang 1990), solid state fermentation methods (Chakraborty and Srinivasan 1993; George et al. 1995; Kaur et al. 2001) and biphasic growth systems (Kaur et al. 2001).

### Cloning and overexpression of alkaline proteases

Microbial strain improvement by either conventional mutagenesis (UV or chemical exposure) or recombinant DNA technology (rDNA) to selectively generate mutants exhibiting higher protease production is also used for improving protease production in microorganisms. Recombinant DNA techniques were instrumental in opening new opportunities for the construction of genetically modified microbial strains with selected enzyme machineries. In this respect, the isolation and cloning of the gene encoding an alkaline protease represents an essential step in the engineering of most efficient producer microorganisms. A number of alkaline protease-encoding bacterial genes have been cloned and expressed in new hosts, the two major organisms of choice for cloning and over-expression being *Escherichia coli* and *B. subtilis* (Table 3).

rDNA technology was used to develop a newly engineered strain of *B. pumilis*, c172-14 (pBX96), by introducing the pBX 96 plasmid (carrying the  $\alpha$ -amylase *amy* gene) into the host strain of alkaliphilic *B. pumilis* c172 by transformation (Feng et al. 2001). The level of alkaline protease production from this new strain was up to 43% improved, compared with the parent strain. The introduction of the pBX 96 plasmid not only changed the

carbon source from glucose to starch, but also got rid of the inhibitory levels of high glucose concentrations on protease production. In another study, Fleming et al. (1995) showed that in vivo recombination can be used in *B. licheniformis* to prepare defined deletions and this deletion of the *spoIIAC* gene gives rise to a sporulation-deficient strain exhibiting extracellular serine protease synthesis. A phenotypic requirement for cysteine was introduced by UV-exposure in a strain of *B. licheniformis* to improve alkaline protease production (Shah et al. 1986). Bierbaum et al. (1994) used a classic mutagenesis approach for the mutation of two protease-producing strains of *B. licheniformis* (strains 4a, 114) to make them insensitive to catabolite repression after UV-exposure, on the basis of increased resistance to the peptide antibiotic nisin.

### Protein engineering: improving stability and catalytic behavior

Protein engineering is the art of altering the properties of a protein by making deliberate changes in its primary structure. This technique has been exploited successfully for obtaining proteases with unique stability and/or substrate specificity at high pH and temperature. It has also contributed substantially towards our understanding of the structure–function relationship of alkaline proteases. Protein engineering allows the introduction of pre-designed changes into the gene for synthesis of a protein with an altered function that is desired for its application. The technique involves two different strategies of random mutagenesis and site-directed mutagenesis (SDM). Generally, these two processes can be applied in combination to develop desired mutants. Random mutagenesis results in large number of variants, but the success of this approach is dependent on an efficient screening procedure, so that variants with an improved and desired property can be identified. However, SDM is dependent on the availability of structural and biochemical data, so that the number of variants to be constructed rationally can be reduced. Further, every protein variant is purified and individually tested for improvement. Alkaline proteases from many microorganisms have been engineered to

**Table 3** Cloning of bacterial alkaline protease genes into suitable hosts for overexpression of proteases. n.s. Not specified

Parent strain	Host strains for cloning and overexpression	Gene	Plasmid vector	X-Fold increase in protease activity	Reference
<i>B. amyloliquefaciens</i>	<i>B. subtilis</i> I-168	Subtilisin	pBS42	200	Wells et al. (1983)
<i>B. subtilis</i> I-168	<i>Escherichia coli</i> and <i>B. subtilis</i> strains	Subtilisin E	pBS42	5	Stahl and Ferrari (1984)
<i>B. amyloliquefaciens</i> ATCC 23844	<i>E. coli</i> strains GX1210, supE thi; <i>B. subtilis</i> strains BR151, IS4	Alkaline and neutral protease	pBD64, pUB110, pGX315; phages M13 mp9, M13 mp8	n.s.	Vasanthan et al. (1984)
<i>B. licheniformis</i> NCIB 6816	<i>E. coli</i> strains HB101, JM101; <i>B. subtilis</i> 168	Subtilisin Carlsberg	pBR322, pUC18, pUC8, pBD64; phages M13 mp8, M13, $\lambda$ 47.1	n.s.	Jacobs et al. (1985)
<i>B. subtilis</i> IFO3013	<i>E. coli</i>	Intracellular protease	pUB110	n.s.	Koide et al. (1986)
<i>B. subtilis</i> var. <i>amylosacchariticus</i>	<i>B. subtilis</i> strains IS1214, 168 and 1012	Subtilisin amylosacchariticus	pHY300PLK	4–20	Yoshimoto et al. (1988)
<i>Bacillus</i> strain YaB	<i>E. coli</i> MC1061, JM109; <i>B. subtilis</i> DB104	Subtilisin (Alkaline elastase YaB)	pUC18, pHY300PLK	17	Kaneko et al. (1989)
<i>B. alcalophilus</i> PB92	<i>B. subtilis</i> 1-A40	Alkaline serine protease	pUB110	1.5	Van der Laan et al. (1991)
<i>Bacillus</i> sp. AH-101	<i>E. coli</i> HB-101	Minor alkaline protease	pHY300PLK	n.s.	Takami et al. (1992a)
<i>Bacillus</i> sp. AH-101	<i>E. coli</i> MV1184, XL 1 blue; <i>B. subtilis</i> DB-104	Thermostable alkaline protease	pUC119, Bluescript II, pAHB1	n.s.	Takami et al. (1992b)
<i>Bacillus</i> sp. 221	<i>E. coli</i> MV1184, XL1 blue; <i>B. subtilis</i> DB104	Alkaline serine protease	pUC119, Bluescript II, pHW1	n.s.	Takami et al. (1992c)
<i>B. stearothermophilus</i> NCIB 10278	<i>E. coli</i> MC1061, JM109; <i>Bacillus subtilis</i> DB104	Subtilisin J	pZ124, pUC18	46	Jang et al. (1992)
<i>Bacillus subtilis</i> DB100 (pUB110) strain	<i>Bacillus subtilis</i> DB100 (pS1) strain	Alkaline protease ( <i>apr</i> )	pS1	3–4	Zaghloul et al. (1994)
<i>Bacillus</i> sp. KSM-K16	<i>B. subtilis</i> SW1214	Alkaline protease	pHY200PLK	n.s.	Hakamada et al. (1994)
<i>Bacillus subtilis</i> ( <i>natto</i> ) No. 16	<i>E. coli</i> DH5 $\alpha$	Structural gene for 90 k serine protease with pI 3.9	pUC118, pUC119	n.s.	Yamagata et al. (1995a)
<i>Bacillus</i> sp. G-825-6	<i>E. coli</i> HB101	Subtilisin sendai gene <i>aprS</i>	pUC118, pUC119	n.s.	Yamagata et al. (1995b)

make them more robust, to suit their utilization with respect to their respective industrial applications.

### SDM and random mutagenesis

SDM has been employed in the construction of subtilisin variants with improved storage and oxidation stabilities. Replacement of an oxidation-sensitive methionine at position 222 by oxidation-resistant amino acids, such as alanine, serine or glutamine, in the linear sequence of *B. amyloliquefaciens* subtilisin BPN' resulted in the prevention of subtilisin inactivation by oxygen bleaches (Estell et al. 1985). Six individual amino acid substitu-

tions (N218S, G169A, Y217K, M50F, Q206C, N76D) at separate positions in the tertiary structure of subtilisin BPN' were found to increase the stability of the enzyme at elevated temperatures (65 °C) and extreme alkalinity (pH 12) (Pantoliano et al. 1989). Under these denaturing conditions, the rate of inactivation of the combination variant was 300 times slower than that of the wild-type subtilisin BPN'. Narhi et al. (1991) reported the stabilization of *apr*-A subtilisin from *B. subtilis* by modification of two Asn–Gly sequences to avoid cyclization. The Asn→Ser substitution in positions 109 and 218 of *apr*-A subtilisin additively stabilized the subtilisin by a total of 7 °C. The introduction of an additional disulfide bond linkage between residues Cys 61 and Cys 98 using pro-

**Table 4** Directed evolution of alkaline proteases

Microbial source	Type of protease	Strategies employed	Change in property	Reference
<i>B. subtilis</i>	Subtilisin E	Error-prone PCR, screening	Activity increased up to 170-fold in dimethylformamide	Chen and Arnold (1993)
<i>B. subtilis</i>	Subtilisin BPN'	Loop removal, cassette mutagenesis, screening	$t^{1/2}$ increased up to 1,000-fold	Strausberg et al. (1995)
<i>B. subtilis</i>	Subtilisin E	Error-prone PCR, screening	Activity increased up to 500-fold in dimethylformamide	You and Arnold (1996)
<i>B. subtilis</i>	Subtilisin E	DNA shuffling, screening	$t^{1/2}$ increased up to 50-fold at 60 °C	Zhao et al. (1998)
<i>B. subtilis</i>	<i>B. lentus</i> subtilisin	Error-prone PCR, enrichment in hollow fibers	50% increase in enzyme secretion	Naki et al. (1998)
<i>B. subtilis</i>	Subtilisin E	Random-priming, screening	Up to 8-fold increase in $t^{1/2}$ at 65 °C	Shao et al. (1998)
<i>B. subtilis</i>	Subtilisin BPN'	Chemical mutagenesis, screening	2-fold increase in enzyme activity at 10 °C	Taguchi et al. (1998)
<i>B. subtilis</i>	Subtilisin E	Error-prone PCR, DNA shuffling, screening	Increased activity at different temperatures and 17 °C rise in $T_m$	Zhao and Arnold (1999)
<i>B. subtilis</i>	Subtilisin S41	Error-prone PCR, cassette mutagenesis, screening	Improved thermostability up to 100-fold	Miyazaki and Arnold (1999)
<i>B. subtilis</i>	Subtilisins	DNA family shuffling, screening	Evolution of multiple properties of enzyme	Ness et al. (1999)

tein engineering resulted in the increased thermostability of subtilisin E (Takagi et al. 1990). Von der Osten et al. (1993) performed protein engineering on two alkaline *Bacillus* proteases, savinase and esperase (commercially produced by Novo Nordisk), to improve the storage stability of the enzymes, lower the isoelectric point of the enzymes (to approach the pH of the liquid detergent formulation, so as to improve their wash performance in laundry detergents) and to improve the thermal stabilities of the enzymes. Aehle et al. (1993), based on a modeled 3-D structure, have designed a model of a highly alkaline subtilisin-like protease (opticlean) from *B. alcalophilus*, using a computer-aided protein design process of "modeling by homology" and starting with the structure of subtilisin Carlsberg 1CSE.BRK from the Brookhaven protein databank, with the aim of increasing the wash performance of the enzyme. A mutant subtilisin E with enhanced thermostability at 60 °C was generated using SDM, by forming a disulfide bridge between two Ser236Cys subtilisin E molecules (Yang et al. 2000a). The random mutagenesis approach on a mutant *M222A* gene was used by same group (Yang et al. 2000b) to develop a thermally stable and oxidation-resistant mutant of subtilisin E. The heat stability of this new mutant was 5-fold greater than that of the wild-type enzyme.

In addition to reports in the literature, several international patents have also been filed on various strategies used by different researchers for obtaining more oxidation-stable mutants of subtilisin. Bech et al. (1993) reported a novel process for stabilizing detergent enzymes for improved stability towards oxidative agents. Subtilisin-309 was modified by replacing the methionine residue at position 222 with a cysteine having a HS group; and this cysteine residue was further modified by replacing the H atom of the HS group of the formula R.

Branner et al. (1996) generated several mutations in the amino acid sequence of matured subtilisin BPN', to construct several subtilisin mutants for use in enzymatic detergent and cleaning compositions. Even though the protein engineering technique is used successfully these days, one earlier report showed that five attempts to stabilize subtilisin BPN' by the introduction of disulfide bonds through SDM failed (Mitchinson and Wells 1989). The disulfides were engineered between residues 26-232, 29-119, 36-210, 41-80 and 148-243. All disulfide bonds formed in the enzyme when the expressed protein was secreted from *B. subtilis* and the disulfides had only minor effects on enzyme kinetics. However, no disulfide mutant was substantially more stable than the wild-type subtilisin BPN'.

### Directed evolution of enzymes

The directed evolution (DE) of enzymes recently emerged as a key technology to generate enzymes with new and improved properties that are of major importance for their industrial applications (Jaeger et al. 2001). This approach has provided a powerful tool for the development of enzymes with novel properties, even without requiring knowledge of enzyme structure and catalytic mechanisms. Arnold (1996), Jaeger et al. (2001), Petrounia and Arnold (2000) and Tobin et al. (2000) have reviewed various aspects and examples of DE approaches for studying key properties of biocatalysts. The DE approach uses three approaches: DNA shuffling, random priming recombination and the staggered extension process (StEP). The DE starts with identification of a target enzyme followed by cloning of the corresponding gene. An efficient expression system is required before

the target gene is subjected to random mutagenesis and/or in vitro recombination, thereby creating molecular diversity. This is followed by screening and identification of enzyme variants with the desired properties. However, use of the DE approach is still far from being widespread or adopted by many research laboratories using standard molecular biology techniques, as it is still unclear at present which strategy is the most efficient for the evolution of a desired property for a given protein (Jaeger et al. 2001). However, some successful attempts have been made to evolve selective biocatalysts, such as lipase, aldolase, hydantoinase, esterase, aspartate aminotransferase, kanamycin nucleotidyl transferase,  $\beta$ -lactamase,  $\beta$ -galactosidase peroxidase, amidase and fucosidase, using the DE approach (Arnold 1996; Jaeger et al. 2001).

Among the few reports on alkaline protease, subtilisin is the enzyme of choice for improving the catalytic behavior of enzymes, using the DE approach. Table 4 summarizes some of the examples of the DE of alkaline proteases. The total activity and organic solvent stability of subtilisin E from *B. subtilis* has been enhanced in aqueous dimethylformamide using the DE approach (You and Arnold 1996). To demonstrate the utility of DE, Zhao et al. (1998) used the StEP process to recombine a set of five thermostabilized subtilisin E variants identified during a single round of error-prone PCR mutagenesis and screening. Screening the StEP-recombined library yielded a subtilisin variant whose half-life was increased 50-fold at 50 °C, compared with wild-type subtilisin. In comparison, Taguchi et al. (1998) used a DE approach, using in vitro random mutagenesis and an improved screening method, to develop a cold-adapted mutant subtilisin from *B. subtilis* UOT0999 with a catalytic efficiency at 10 °C 100% higher than the wild-type strain. In another report, the thermal stability of subtilisin E was increased by converting it into a thermitase using DE (Zhao and Arnold 1999). This fascinating area of protein design will no doubt be the heart of future commitments in the enzyme industry.

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### **Solvent and enzyme engineering**

Studies over the past two decades have revealed that, using the hostile environment provided to them in organic solvents, enzymes can catalyze reactions that were impossible in water, become more stable and exhibit new behavior such as “molecular memory” (Klibanov 2001). The stability of proteins and enzymes is a major concern for their industrial applications. Biocatalysts with high thermostability have a prolonged viability. For many biocatalysts, enzymatic reactions and stability of enzyme at high temperatures are pre-requisites for industrial use. However, the stability of enzyme in organic solvents, at extreme pH and pressure and stability towards mechanical disturbances are required for organic synthesis, chemical analysis, isolation and purification of chemicals, in therapeutics and diagnostics and in the study of protein structures and functions (Wong and Wong 1992).

Thus, a consistent search for methods to prepare stable enzymes is necessary. Some of the examples of methods used for stabilizing alkaline proteases are chemical modification using PEG (Inada et al. 1986), bio-imprinting of  $\alpha$ -chymotrypsin in anhydrous media (Stahl et al. 1991), chemical crosslinking (Wong and Wong 1992), molecular imprinting (Russell and Klibanov 1988), immobilization in hydrophobic solvents (Kise et al. 1990), use of lyoprotectants [such as sugars, substrate-resembling ligands (Dabulis and Klibanov 1993) and crown ethers (Santos et al. 2001)] and protein engineering (Pantoliano et al. 1989; You and Arnold 1996). In view of the large number of research papers concerning the organic-solvent stability of alkaline proteases and peptide synthesis, only a few chosen methods have been adopted by industry. The primary reason being that the rejected biocatalyst does not have either of these two properties: substrate specificity, or sufficient organic-solvent stability. Thus, the discovery of novel proteases having all the required properties is essential for the use of enzymes on an industrial scale.

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### **Discovering novel proteases**

Looking into the depth of microbial diversity, there is always a chance of finding microorganisms producing novel enzymes with better properties and suitable for commercial exploitation. The multitude of physicochemically diverse habitats has challenged nature to develop equally numerous molecular adaptations in the microbial world. Microbial diversity is a major resource for biotechnological products and processes.

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### **Cloning of the soil metagenome: a different approach**

Over billions of years, prokaryotic microbes developed a wealth of physiologies and molecular adaptations that enabled their survival in virtually every environmental niche, some so extreme and inhospitable that no other life forms could coexist. The versatility and adaptive power of the prokaryotic design was such that, with their evolutionary headstart, the bacterial and archaeal kingdoms produced a degree of organism and molecular diversity unparalleled in nature. Natural and in particular microbial biodiversity is our planet's greatest but least-developed resource for biotechnological innovation. The biosphere is dominated by microorganisms, yet to date most microbes in nature have not been studied. This is mainly due to the fact that historically the only way to reliably characterize a microorganism was by isolation, purification and fermentation, to be able to score its biochemical and physiological features on the “macroscopic” level of a pure culture. Incidentally, the vast majority of microbes, often thousands of species in a single environmental niche like soil (Torsvik et al. 1990), cannot be grown in the laboratory; and it is estimated that, on aver-

age, less than 1% have ever been identified (Amann et al. 1995). Traditional methods for culturing microorganisms fail to represent the scope of microbial diversity in nature and they limit analysis to those that grow under laboratory conditions (Hugenholtz et al. 1998; Rondon et al. 2000).

The advent of powerful analytical tools like PCR and the recognition of molecular markers like the 16S ribosomal DNA as species-specific identification-tags have provided us with a truly “microscopic” sensitivity down to single-cell detection, thereby widening our view of natural biodiversity tremendously. The recent surge of research in molecular microbial ecology provides compelling evidence for the existence of many novel types of microorganisms in high numbers and varieties; and it provides an entirely different approach for tapping into the potentially limitless resource of uncultured bacteria. To collectively target all microbial genomes present in a given habitat is the goal of modern enzyme discovery programs. Handelsman and coworkers (1998) coined the term “metagenome” to describe this DNA resource. Thereby, the genomic DNA of the entire microbial consortia is extracted (Krsek and Wellington 1999; Zhou et al. 1996) and laid down in complex recombinant DNA libraries for subsequent screening programs. The direct DNA extraction and cloning strategy to discover novel enzymes is feasible and has made an impact in the recent literature (Cottrell et al. 1999; Henne et al. 1999, 2000; Radomski et al. 1998; Rondon et al. 2000). The list of reported enzyme activities discovered this way (lipase, esterase, amylase, nuclease, chitinase, xylanase) is still rather small, but will undoubtedly grow rapidly. The only reported screen for protease activity in metagenome libraries so far was by the Handelsman group (Rondon et al. 2000), although with a negative outcome.

We have activity-screened a small (100,000 primary colony-forming units, 8–12 kbp insert size) metagenomic library prepared in pUC18 from DNA isolated from an alkaline loessian soil sampled near Heidelberg, Germany (Lorenz et al. 2001). Plated on skimmed-milk agar, a colony producing a clearing zone was isolated and its metagenomic DNA insert was analyzed. The insert apparently harbored an EDTA-sensitive 30-kDa metalloprotease of 275 amino acid residues and an apparent pI of 8.98. The sequence contained the prototypical HEXxH-signature of metalloproteases, yet the highest similarity to any entry in the databases was only 30%, the most similar protease being an enzyme from starfish (Accession number S71352). There can be no doubt that, with time, novel alkaline serine proteases will also be discovered this way. Despite the enormous resource of molecular diversity present in metagenomic DNA, the number of actual discoveries will be limited by technological and logistic constraints. For a complete representation of a metagenome, as present in soil, Handelsman (Handelsman et al. 1998) estimates that in the order of  $10^6$  bacterial artificial chromosome clones with an average insert size of 100 kbp will be necessary. Libraries of this size are not trivial to construct, archive or screen ap-

propriately. Cloning of metagenomic DNA will naturally be in heterologous hosts, essentially in *E. coli*, for the sake of achieving the high clone numbers necessary to get anywhere near a representation of the microbial diversity present in the metagenome and downstream in *Bacillus* species or *Streptomyces lividans*, for the sake of optimizing gene expression. Although activity screening based on the functional expression of enzymes is attractive, as it has the potential to yield novel molecular entities unrelated to known sequence space, problems associated with heterologous gene expression will naturally limit its success. However, our experience is that molecular diversity in metagenomic DNA is so great that even inherently conservative sequence-based screening approaches may yield functional and novel molecular entities with sequence identities as low as 40% to any known genes in the public databases (Lorenz et al. 2001; unpublished data). Clearly therefore, both sequence-based and activity-based screening approaches will be necessary to gain full advantage of the molecular treasures retrieved “from the dirt”.

Thus, the above-accumulated evidence provides a window into a world of microbial diversity that is astonishing in its magnitude and breadth and is open to tap the vast genetic and biochemical potential of microorganisms to obtain products and processes of biotechnological value. The metagenome approach thus provides a means to view both the structural and the functional genomics of microbial diversity and pave a pathway to search out and discover novel genes for obtaining newer and useful industrial alkaline proteolytic enzymes with better properties.

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### Applications of alkaline proteases

Alkaline proteases account for a major share of the enzyme market all over the world (Godfrey and West 1996; Kalisz 1988). Alkaline proteases from bacteria find numerous applications in various industrial sectors and different companies worldwide have successfully launched several products based on alkaline proteases (Table 1). The success of detergent enzymes has led to the discovery of a series of detergent proteases with specific uses. Alkazym (Novodan, Copenhagen, Denmark) is an important enzyme for the cleaning of membrane systems. Other enzymes used for membrane cleaning are Terga-zyme (Alconox, New York, USA), Ultrasil (Henkel, Dusseldorf, Germany) and P3-pardigm (Henkel-Ecolab, Dusseldorf, Germany). Pronod 153L, a protease enzyme-based cleaner is used to clean surgical instruments fouled by blood proteins. Subtilopeptidase A is an enzyme-based optical cleaner, presently marketed in India (Kumar et al. 1998). Sakiyama et al. (1998) reported the use of a protease solution for cleaning the packed columns of stainless steel particles fouled with gelatin and  $\beta$ -lactoglobulin. In addition to these major applications, alkaline proteases are also used to a lesser extent for other applications, such as contact lens cleaning (Nakagawa

1994), molecular biology for the isolation of nucleic acid (Kyon et al. 1994), pest control (Kim et al. 1999), degumming of silk (Kanehisa 2000; Puri 2001) and selective delignification of hemp (Dorado et al. 2001), which all may be technically interesting, but have not reached commercial success in terms of impressive sales figures. The bulk uses of alkaline proteases in industrial sectors are described in the following section.

## Food and feed industry

Traditionally, microbial proteases have been exploited in the food industries in many ways. Alkaline proteases have been used in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks (Neklyudov et al. 2000; Ward 1985). The basic function of proteases is to hydrolyze proteins; and this property has been exploited for the preparation of protein hydrolysates of high nutritional value. The alkaline proteases are used in hydrolysate production from various natural protein substrates. The commercial protein hydrolysates are derived from casein (Miprodan; MD Foods, Viby, Germany), whey (Lacprodan; MD Foods) and soy protein (Proup; Novo Nordisk, Bagsvaerd, Denmark). Rebeca et al. (1991) reported the production of fish hydrolysates of high nutritional value, using *B. subtilis* proteases. Matsui et al. (1993) reported on protease hydrolysates having angiotensin-I-converting enzyme-inhibitory activity from sardine muscle obtained after treatment with *B. licheniformis* alkaline protease. Fujimaki et al. (1970) used alkaline protease for the production of soy protein hydrolysates. Cheese whey is an abundant liquid by-product of the dairy industry with an estimated world production of  $145 \times 10^6$  t/year. Perea et al. (1993) used alkaline protease for the production of whey protein hydrolysate, using cheese whey in an industrial whey bioconversion process. O'Meara and Munro (1984) reported the up-grading of lean meat waste to edible products by alkaline protease hydrolysis of the meat waste, using commercial alkaline proteases. Ohmiya et al. (1979) reported the use of immobilized alkaline protease (on Dowex MWA-1 by gluteraldehyde) in cheese-making. Tanimoto et al. (1991) reported the use of alkaline protease in the enzymatic modification of zein to produce a non-bitter peptide fraction with high Fischer ratio for patients with hepatic encephalopathy.

Keratinolytic activity of alkaline protease has also been exploited in the production of proteinaceous fodder from waste feathers or keratin-containing materials. Dalev (1990, 1994) and Cheng et al. (1995) reported the use of alkaline proteases (B72 from *B. subtilis* and *B. licheniformis* PWD-1) for the hydrolysis of feather keratin, to obtain a protein concentrate for fodder production.

## Peptide synthesis

Since the first report of Bergman and Frankel-Conrat (1937), on protease-catalyzed peptide synthesis using the reverse-enzymatic reaction of hydrolysis, the proteases have frequently been used for peptide synthesis (Clapes et al. 1997; Isono and Nakajima 2000; Kise et al. 1990; Morihara 1987). Enzymatic peptide synthesis offers several advantages over chemical methods, e.g. reactions can be performed stereospecifically and reactants do not require side-chain protection, increased solubility of non-polar substrates, or shifting thermodynamic equilibria to favor synthesis over hydrolysis. There is less need for expensive protecting-groups, organic solvents, or hazardous chemicals, resulting in production costs competitive with those of chemical methods (Morihara 1987). However, the major limitation for the use of protease in synthetic chemistry is the strongly reduced activity of the enzyme under anhydrous conditions. Proteases have been used successfully for the synthesis of dipeptides (Barros et al. 1999) and tripeptide (So et al. 2000), regioselective sugar esterification (Riva et al. 1988) and dia-stereoselective hydrolysis of peptide esters (Chen et al. 1991b). A number of reports are available on the use of alkaline protease in peptide synthesis and the resolution of racemates of amino acids. The nature and type of organic solvent have a strong effect on protease activity in organic solvents (Kawashiro et al. 1997). Castro (1999) studied the effect of 14 neat organic solvents on the enzymatic activity of subtilisin and reported that subtilisin showed 500-fold higher activity in glycerol compared with ethylene glycol, *N*-methylformamide, 1,2- and 1,3-propanediol. Nagashima et al. (1992) and Gololobov et al. (1994) studied the effect of enzyme, solvent, medium and substrate reactions, using  $\alpha$ -chymotrypsin, subtilisin BPN' and subtilisin Carlsberg from *B. subtilis* strain 72 on peptide synthesis in organic solvents. An industrial protease, neutrase (co-deposited with sorbitol onto polyamide) was used for the synthesis of several  $N^\alpha$ -protected dipeptide derivatives in acetonitrile (Clapes et al. 1997). Also, Chen et al. (1991a) reported the kinetic resolution of *N*-protected amino acid esters in organic solvents catalyzed by an industrial alkaline protease, alcalase. Sutar et al. (1992) used alkaline protease for the resolution of DL-phenylalanine and DL-phenylglycine. The proteinase from an extremophile, *Thermus* Rt41A, immobilized on controlled-pore glass beads, was used for peptide synthesis, using the synthesis of Bz-Ala-Tyr-NH<sub>2</sub> as a model system (Wilson et al. 1994). In order to study the dependence of subtilisin's enantioselectivity on organic solvents, a different approach, computer-assisted modeling, was used to study the asymmetric transesterifications of achiral esters with chiral alcohols catalyzed by subtilisin Carlsberg in anhydrous organic solvents (Fitzpatrick et al. 1992). The models of binding derived from computer-simulated data predicted changes in subtilisin enantioselectivity. Okazaki et al. (2000) described the use of a surfactant-protease complex as a

novel biocatalyst for peptide synthesis in hydrophilic organic solvents.

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### Leather industry

The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental pollution and safety hazards. Thus, for environmental reasons, the biotreatment of leather using an enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction, thus being ecofriendly (Andersen 1998). Alkaline proteases with elastolytic and keratinolytic activity can be used in leather-processing industries. Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced. Bating is traditionally an enzymatic process involving pancreatic proteases. However, recently, the use of microbial alkaline proteases has become popular (Varela et al. 1997). Alkaline proteases speed up the process of dehairing, because the alkaline conditions enable the swelling of hair roots; and the subsequent attack of protease on the hair follicle protein allows easy removal of the hair. Varela et al. (1997) reported the use of *B. subtilis* IIQDB32 alkaline protease for unhairing sheepskin. George et al. (1995) used *B. amyloliquefaciens* alkaline protease for unhairing hides and skins. Hameed et al. (1996, 1999) used *B. subtilis* K2 alkaline protease in bating and leather processing.

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### Management of industrial and household waste

Proteases solubilize proteinaceous waste and thus help lower the biological oxygen demand of aquatic systems. Recently, the use of alkaline protease in the management of wastes from various food-processing industries and household activities opened up a new era in the use of proteases in waste management. Dalev (1994) used alkaline protease from *B. subtilis* for the management of waste feathers from poultry slaughterhouses. Waste feathers make up approximately 5% of the body weight of poultry and are considered to be a high protein source for food and feed, provided their rigid keratin structure is completely destroyed. The use of keratinolytic protease for food and feed industry waste, for degrading waste keratinous material from poultry refuse (Ichida et al. 2001) and as depilatory agent to remove hair from the drains (Takami et al. 1992d) has been reported. A formulation containing proteolytic enzymes from *B. subtilis*, *B. amyloliquefaciens* and *Streptomyces* sp. and a disulfide reducing agent (thioglycolate), that enhances hair degradation and helps in clearing pipes clogged with hair-containing deposits, is currently available in market. It was prepared and patented by Genex (Jacobson et al. 1985).

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### Photographic industry

Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery. These waste films contain 1.5–2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Conventionally, this silver is recovered by burning the films, which causes undesirable environmental pollution. Furthermore, base film made of polyester cannot be recovered using this method. Since the silver is bound to gelatin, it is possible to extract silver from the protein layer by proteolytic treatments. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled. Alkaline protease from *B. subtilis* decomposed the gelatin layer within 30 min at 50–60 °C and released the silver (Fujiwara et al. 1989). Ishikawa et al. (1993) have reported the use of alkaline protease of *Bacillus* sp. B21-2 for the enzymatic hydrolysis of gelatin layers of X-ray films to release silver particles. The alkaline proteases of *Bacillus* sp. B18' (Fujiwara et al. 1991) and *B. coagulans* PB-77 (Gajju et al. 1996) were also efficient in decomposing the gelatinous coating on used X-ray films from which the silver could be recovered.

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### Medical usage

Alkaline proteases are also used for developing products of medical importance. Kudrya and Simonenko (1994) exploited the elastolytic activity of *B. subtilis* 316M for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses. Kim et al. (1996) reported the use of alkaline protease from *Bacillus* sp. strain CK 11-4 as a thrombolytic agent having fibrinolytic activity.

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### Silk degumming

One of the least explored areas for the use of proteases is the silk industry and only a few patents have been filed describing the use of proteases for the degumming of silk (Kanehisa 2000). Sericin, which is about 25% of the total weight of raw silk, covers the periphery of the raw silk fibers, thus providing the rough texture of the silk fibers. This sericin is conventionally removed from the inner core of fibroin by conducting shrink-proofing and twist-setting for the silk yarns, using starch (Kanehisa 2000). The process is generally expensive and therefore an alternative method suggested is the use of enzyme preparations, such as protease, for degumming the silk prior to dyeing. In a recent study in our laboratory (Puri 2001), the silk-degumming efficiency of an alkaline protease from *Bacillus* sp. RGR-14 was studied and results were analyzed gravimetrically (fiber weight reduction) and by scanning electron microscopy

(SEM) of treated silk fiber. After 5 h of incubation of silk fiber with protease from *Bacillus* sp., the weight loss of silk fiber was 7.5% (Puri 2001). SEM of the fibers revealed that clusters of silk fibers had fallen apart as compared with the smooth and compacted structure of untreated fiber.

### Proteases in the detergent industry: an overview

Enzymes have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies. Applications of detergent proteases have grown substantially and the largest application is in household laundry detergent formulations. The increased reliance of detergent manufacturers on enzyme technology is because of consumer-recognizable cleaning benefits, the addition of completely new performance benefits, fabric restoration and an increased performance/cost ratio, because of the availability of more efficient enzymes and the industry trend toward reduced pricing. Current market trends and consumer needs are influencing the development of enzymes for detergent applications, with the emphasis on enzymes that have improved performance/cost ratios, increased activity and improved compatibility with other detergent ingredients. In addition, enzyme suppliers and detergent manufacturers are actively pursuing the development of new enzyme activities that address the consumer-expressed need for improved cleaning, fabric care and antimicrobial benefits. However, apart from their use in laundry detergents, they are also popular in the formulation of household dishwashing detergents and both industrial and institutional cleaning detergents (Godfrey and West 1996; Showell 1999).

### History

The history of detergent enzymes dates back to 1914, when two German scientists, Rohm and Haas, used pancreatic proteases and sodium carbonate in washing detergents. The product was named Burnus, after the white arab cloak. The first detergent containing the bacterial enzyme was introduced into the market in 1956 under the trade name Bio-40. However, it was only in 1963 when an alkaline protease, alcalase, was effectively incorporated in detergent powder and was marketed by Novo Industry, Denmark under the trade name Biotex. Unfortunately, detergent proteases faced a setback in the early 1970s, due to unfavorable publicity when some workers developed an allergic reaction during the handling of these enzymes. This problem was solved by the introduction of dust-free encapsulated products. Today, detergent enzymes account for 89% of the total protease sales in the world; and a significant share of the market is captured by subtilisins and/or alkaline proteases from

many *Bacillus* species. The detergent enzyme market has grown nearly 10-fold during the past 20 years. In the 1980s and early 1990s, the major market share (>55%) of the detergent enzyme was held by Gist-Brocades in The Netherlands, Genencor International in the United States, Solvay in Belgium and Showa-Kenko in Japan. These suppliers marketed a full range of enzymes for liquid and powder detergents. Beginning in 1995, however, there was considerable need for rationalization in the detergent enzyme industry, owing to the relatively high cost of manufacturing, coupled with increased pressure from detergent manufacturers to drive down raw material costs. Genencor International purchased the detergent enzyme businesses of Gist-Brocades and Solvay; and Novo Nordisk acquired Showa-Denko's detergent enzyme business. Today, Novo Nordisk and Genencor International are the major suppliers of detergent enzymes, supplying up to 95% of the global market of proteases.

### Selection and evaluation of detergent protease performance

One of the important parameters for selection of detergent proteases is the pI value. It is known that detergent proteases perform best when the pH value of the detergent solution in which it works is approximately the same as the pI value for the enzyme. However, there are many more parameters involved in the selection of a good detergent protease, such as compatibility with detergent components, e.g. surfactants, perfumes and bleaches (Bech et al. 1993; Gupta et al. 1999; Kumar et al. 1998), good activity at relevant washing pH and temperature (Aehle et al. 1993; Beg et al. 2002b; Oberoi et al. 2001), compatibility with the ionic strength of the detergent solution, stain degradation and removal potential, stability and shelf life (Showell 1999). Over the past 30 years, the proteases in detergents have changed from being minor additives to being the key ingredients. There is always a need for newer enzymes with novel properties that can further enhance the wash performance of currently used enzyme-based detergents. Conventionally, detergents have been used at elevated washing temperatures, but at present there is considerable interest in the identification of alkaline proteases which are effective over a wide temperature range (Oberoi et al. 2001). In addition, the current consumer demands and increased use of synthetic fibers, which cannot tolerate high temperatures, have changed washing habits towards the use of low washing temperatures (Hasan and Tamiya 1997; Kitayama 1992; Nielsen et al. 1981). This has pushed enzyme manufacturers to look for novel enzyme that can act under low temperatures. Novo Nordisk Bioindustry in Japan has developed a detergent protease called Kannase, which keeps its high efficiency, even at very low temperatures (10–20 °C). Further, a good detergent enzyme should also be stable in the presence of oxidizing agents and bleaches. In general, the majority of the

commercially available enzymes are not stable in the presence of bleaching/oxidizing agents. Hence, the latest trend in enzyme-based detergents is the use of recombinant DNA technology to produce bioengineered enzymes with better stability. Bleach- and oxidation-stability has been introduced through SDM and protein engineering by the replacement of certain amino acid residues (Bech et al. 1993; Estell et al. 1985; Wolff et al. 1996; Yang et al. 2000b).

The evaluation of detergent proteases is mainly dependent upon parameters such as the pH and ionic strength of the detergent solution, the washing temperature and pH, mechanical handling, level of soiling and the type of textile. In the case of laundry detergents, protease performance is evaluated by using soiled test-fabrics and the efficiency is measured either visually or by measuring the reflectance of light under standard conditions (Durham et al. 1987; Masse and Tilburg 1983; Nielsen et al. 1981; Wolf et al. 1996).  $\Delta R$  is the measure of efficiency and is defined as the difference in reflectance between fabric treated with and without enzyme. In a recent study by our group, the wash performance analysis of a SDS-stable alkaline protease from *Bacillus* sp. RGR-14 (Oberoi et al. 2001) was studied using a reflectance meter. Wash performance analysis of RGR-14 protease on grass- and blood-stained cotton fabrics showed an increase in reflectance (14% with grass stains, 25% with blood stains) after enzyme treatment. However, enzyme in conjunction with detergent proved best, with a maximum reflectance change of 46% and 34% for grass and blood stain removal, respectively, at 45 °C. Stain removal was also effective after protease treatment at 25 °C and 60 °C.

## Conclusions and future prospects

Several aspects of alkaline proteases have stimulated research on the study of biochemical, regulatory and molecular aspects of proteolytic enzyme systems (Rao et al. 1998). Looking into the commercial success of this enzyme class, researchers have now started aiming at the discovery and engineering of novel enzymes that are more robust with respect to their pH and temperature kinetics, using techniques of protein engineering and the identification of active site residues through chemical modifications, X-ray crystallographic data and SDM. In future, protein engineering will offer possibilities of generating proteases with entirely new functions. Hence, although microbial alkaline proteases already play an important role in several industries, their potential is much greater and their applications in future processes are likely to increase in the near future. The pursuit of other, newer approaches, such as novel discovery strategies targeting new dimensions of molecular diversity and technologies to improve performance characteristics by in vitro evolutionary changes of protein primary structures will certainly be the major field of development in next few years.

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