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REVIEW ARTICLE

Search for novel proteolytic enzymes aimed at textile and agro-industrial applications: An overview of current and novel approaches

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Abstract
The types and sources of proteolytic enzymes, enzyme assays, strategies for fermentation yield improvement, and novel proteases and their applications in industrial sectors are widely covered in this review. We give a special focus on alkaline proteases for the textile and detergent industries, as well as for the degradation of keratin-rich wastes.

Keywords: *wool, casein, biocatalyst, metagenomic, extremophile, response surface methodology*

Introduction

Proteolytic enzymes are ubiquitous; they are found in all living organisms and are essential for cell growth and differentiation. However, only those producing substantial amounts of extracellular protease(s) have been exploited commercially (Gupta et al. 2002b), and microorganisms, especially strains of *Bacillus* sp., currently dominate the industrial sector (Fujinami & Fujisawa 2010; Rao et al. 1998).

Various isolation methods have been described and discussed that enable screening and selection of promising organisms for industrial production. In addition, there are many possibilities for modifying biocatalysts through molecular approaches. For instance, improvement using mutagenesis and/or recombinant DNA technology has been applied to increase the efficiency of the producer strain. The search for microbial sources of novel alkaline proteases using metagenomic approaches has also revealed a wide molecular diversity (Gupta et al. 2002b). These fascinating developments will eventually allow the biotechnological exploitation of uncultured microorganisms, which far outnumber the species accessible by cultivation (regardless of their original habitat).

To develop efficient enzyme-based processes suitable for industry, prior knowledge of various fermentation parameters, purification strategies and properties of the biocatalyst itself are of the utmost importance. Furthermore, the assay method, including selection of substrate and analytical protocol, depends on the intended industrial application. While a large array of assays are available in the literature, with the advent of molecular approaches aimed at producing better biocatalysts, less conventional substrates and assay protocols have become increasingly important – chiefly those that can be conducted at micro/nano-scale (Gupta et al. 2002a).

Production of proteases in bioreactors is usually mastered by varying the C/N ratio, and the presence and level of metabolizable sugars, for example, glucose. Successful scale-up has been achieved using fed-batch, continuous and chemostat approaches, by extending the stationary phase of the culture (Joshi et al. 2008). Conventional purification strategies include concentration by bulk chromatography,

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or aqueous two-phase systems (solid/liquid via precipitation, or liquid/liquid via solvent extraction).

Keratinases form a unique group of proteolytic enzymes that display the ability to degrade the insoluble protein, keratin, which is composed of extensively cross-linked structural polypeptides and is recalcitrant to most proteases (Gupta & Ramnani 2006). Such enzymes are often produced in the presence of keratinous substrates, for example, hair, feather, wool, nail and horn. They form a class of particularly robust enzymes, with wide temperature and pH activity ranges; most of them are serine, or metallo-proteases. Sequence homologies exhibited by keratinases have indicated their relatedness to the subtilisin family (Adıgüzel et al. 2009).

Keratinases find applications in feather by-product upgrading, towards feed and fertilizer formulations, in the detergent and leather industries, where they serve as speciality enzymes (Gupta & Ramnani 2006), and also in wool and silk cleaning. Their enhanced dehairing potential has led to development of less polluting hair-saving and dehairing technology in the leather industry, as well as of personal care products in the cosmetic industry.

Types and sources of proteolytic enzymes

Proteolytic enzymes (or peptidases, a synonymous term recommended by NC-IUBMB) constitute a class of hydrolases that can hydrolyse peptide bonds, thus forming a distinct subclass, EC 3.4. Peptidases are further sub-divided into exopeptidases (EC 3.4.11–19), which act only near the termini of polypeptide chains, and endopeptidases (EC 3.4.21–24 and EC 3.4.99), which act preferentially away from termini (Table I). As a consequence, different proteins can be regarded as distinct peptidases, even when they express similar activities (Rao et al. 1998; Beynon & Bond 2001).

Recently, a third mode of classification, MEROPS, has been proposed, which attempts to group peptidases according to structural features and evolutionary relationships underlying those features (Rawlings et al. 2010). In the MEROPS system, each peptidase is assigned to a family on the basis of statistically significant similarities in its amino acid sequence, and families thought to be homologous are, in turn, grouped together in a clan (Rawlings et al. 2010).

Due to their metabolic and commercial importance, there is a vast literature on the biochemical and biotechnological aspects of peptidases, including animal, plant or microbial sources (Anwar & Saleemuddin 1998; Rao et al. 1998; Kumar & Takagi 1999; Niehaus et al. 1999; Demirjian et al. 2001; Gupta et al. 2002b; Antão & Malcata 2005; Gupta & Ramnani 2006; Dubey et al. 2007; Brandelli 2008; Klomklao 2008; Esposito et al. 2009; Brandelli et al. 2010; Fujinami & Fujisawa

Table I. EC system of classification of peptidases, according to latest full publication list (Enzyme Nomenclature, 1992) and amended by 17 supplements (adapted from www.chem.qmul.ac.uk/iubmb/enzyme, accessed on March 2011).

Sub-subclass	Type of peptidase	Mode of action	Number of entries
3.4.11–19	Exopeptidases	Acting only near the ends of polypeptide chains	80
		At a free N-terminus	
3.4.11	Aminopeptidases	Liberating a single amino acid residue	21
3.4.14	Dipeptidyl-peptidases	Liberating a dipeptide or a tripeptide	9
		At a free C-terminus	
3.4.15	Peptidyl-dipeptidases	Liberating a dipeptide	4
3.4.16–18	Carboxypeptidases	Liberating a single amino acid residue	
3.4.16	Serine-type carboxypeptidases	—	4
3.4.17	Metallocarboxypeptidases	—	20
3.4.18	Cysteine-type carboxypeptidases	—	1
		Specific for dipeptides	
3.4.13	Dipeptidases	—	12
		Removing terminal residues that are substituted, cyclized or linked by isopeptide bonds	
3.4.19	Omega peptidases	—	9
3.4.21–25 + 3.4.99	Endopeptidases	Cleaving internal bonds in polypeptide chain	280
3.4.21	Serine endopeptidases	—	99
3.4.22	Cysteine endopeptidases	—	58
3.4.23	Aspartic endopeptidases	—	40
3.4.24	Metallo endopeptidases	—	81
3.4.25	Threonine endopeptidases	—	2
3.4.99	Endopeptidases of unknown type	—	0

2010). Plants produce many useful peptidases, viz. papain (Dubey et al. 2007), bromelain (Benucci et al. 2011) and ficain (Devaraj et al. 2008), with applications in medicine and the food industry. However, the use of plants as sources of commercially relevant peptidases is typically constrained by availability of land for cultivation and suitability of weather conditions for growth (Rao et al. 1998). Moreover, production of proteases from plants is a time-consuming process (Rao et al. 1998). The most commonly used peptidases of animal origin are gastrointestinal varieties, for example, pepsin, trypsin, chymotrypsin and rennin (Klomklao 2008; Esposito et al. 2009; Mendes et al. 2009). These peptidases are prepared in pure form to large amounts, but their production rate is critically dependent on the slaughter of livestock, which may be limited by political and agricultural policies (Rao et al. 1998).

Microorganisms from all three domains of life – Eukarya, Bacteria and Archae (Brandelli et al. 2010), are a much more favourable source for industrial applications owing to their much broader biochemical diversity, ease of genetic manipulation and ability to be cultured on a large scale (Gupta et al. 2002b). These microorganisms have been isolated from a number of habitats, ranging from Antarctic soils (Marshall 1998) to hot springs (Pillai & Archana 2008), and from acidic (Yallop et al. 1997) to extremely alkaline environments (Singh et al. 1999; Genckal & Tari 2006), including both aerobic and anaerobic ecosystems. Isolation from harsh environments can be used to discover peptidases capable of withstanding similarly harsh industrial conditions. A number of reviews have focused on comparative analysis, emphasizing the microbial diversity among peptidase producers (Anwar & Saleemuddin 1998; Kumar & Takagi 1999; Niehaus et al. 1999; Gupta et al. 2002b; Haki & Rakshit 2003; Gupta & Ramnani 2006; Brandelli 2008; Brandelli et al. 2010). Information on the most relevant genera is summarized in Table II, particularly those proteolytic enzymes that have emerged as useful in textile and agro-industrial processing.

A significant fraction of commercially available bacterial peptidases is produced by the *Bacillus* genus (Rao et al. 1998; Gupta et al. 2002b); these are predominantly neutral and alkaline. This should come as no surprise, since those species are known for their wide metabolic versatility including the ability to produce highly resistant dormant endospores, which has allowed them to survive in extreme environments (Alcaraz et al. 2010). In particular, several *Bacillus* spp. have recently been shown to synthesize highly thermostable and alkaline peptidases (Haddar et al. 2009; Cheng et al. 2010; Fujinami & Fujisawa 2010; Rachadech et al. 2010; Shrinivas & Naik 2011), which makes them useful for textile and agro-industrial applications, e.g. detergent additives, leather processing, silk degumming and wool finishing, as well as general purpose

Table II. Genera reported encompassing microbial species able to produce proteolytic enzymes with potential industrial applications (adapted from Demirjian et al. 2001; Breithaupt 2001; Niehaus et al. 1999; Brandelli 2008; Brandelli et al. 2010; Gupta & Ramnani 2006; Anwar & Saleemuddin 1998; Kumar & Takagi 1999; Gupta et al. 2002; Haki & Rakshit 2003).

Eukarya	Bacteria		Archae
Thermonospora	*Gram positive*	*Gram negative*	*Pyrococcus Staphylothermus*
Engyodontium (formerly *Tritirachium*)			
Conidiobolus	*Bacillus*	*Vibrio*	*Thermococcus*
Aspergillus	*Lysobacter*	*Xanthomonas*	*Sulfolobus*
Doratomyces	*Nesternokia*	*Stenotrophomonas*	*Desulfurococccus*
Myrothecium	*Kocuria*	*Chryseobacterium*	*Halobacterium*
Paecilomyces	*Microbacterium*	*Thermotoga*	*Pyrobaculum*
Seopulariopsis	*Kurthia*	*Pseudomonas*	
Thrichoderma	*Thermoanaerobacter*	*Fervidobacterium*	
Thricophyton	*Clostridium*	*Alcaligenes*	
Cephalosporium	*Kytococcus*	*Janthinobacterium*	
Chrysosporium	*Nocardiopsis*	*Halomonas*	
Entomophthora	*Streptomyces*	*Thermus*	
Fusarium	*Thermoactinomyces*		
Penicillium	*Coprothermobacter*		
Rhizopus	*Microbispora*		
Scedosporium	*Terrabacter*		
Dendryphiella	*Oerskovia*		
Scolebasidium	*Arthrobacter*		
Candida			
Yarrowia			
Aureobasidium			
Malbranchea			
Torula			

formulations of food and feed. In fact, among alkalophiles, *Bacillus* species and their extracellular peptidases have been the focus of several studies because of their non-pathogenicity (except for the *Bacillus cereus* species) and ability to secrete peptidases across their single membrane system directly into the culture medium (Gupta et al. 2002b; Burg 2003; Fujinami & Fujisawa 2010). Despite the advantages of *Bacillus* secretory systems (Westers et al. 2004; Araújo et al. 2008; Vavrová et al. 2010), their use to express heterologous proteins has a number of shortcomings. Their intrinsic cellular control systems for removal of misfolded or incompletely synthesized proteins can represent a bottleneck for production of heterologous proteins to significant levels (Li et al. 2004; Zweers et al. 2008).

Fungi synthesize a wider variety of proteolytic enzymes than do bacteria, including acid, neutral and alkaline peptidases (Rao et al. 1998). Even though fungal peptidases (mainly keratinases) with interesting biochemical properties are produced by non-dermathophytic fungi (Brandelli et al. 2010), this group has attracted little commercial interest, probably because fungal peptidases exhibit low reaction rates and poor heat tolerance when compared with their bacterial counterparts (Rao et al. 1998).

Keratinases (EC 3.4.21–24) constitute a special group among peptidases that have the ability to degrade insoluble keratin substrates (Gupta & Ramnani 2006; Brandelli 2008). Potential applications have been claimed with uses in such traditional industrial sectors as detergent, leather and feed, but also in newer fields, viz. prion degradation, biodegradable plastic manufacture and feather meal production (Onifade et al. 1998; Gupta & Ramnani 2006). Thermophilic and alkalophilic microorganisms (mainly Bacteria and Archae, see Table II) are thus of great interest for industrial textile and agro-processing entailing keratin degradation, especially because the process is facilitated by high temperature and pH (Brandelli et al. 2010).

Commercially available peptidases for industrial applications are derived primarily from *Bacillus* spp. However, there is demand from the industrial side for novel proteases, which is not being matched by the supply capacity of most enzyme-producing companies. The vast majority of enzymes available have limited stability in extreme environments (pH, temperature and organic solvents), which restrict their biotechnological applications (Breithaupt 2001; Mansfeld & Ulbrich-Hofmann 2007). Current attempts to address these limitations will be dealt with below, as part of application-oriented research programs.

Research strategies of enzyme companies have a major focus on the improvement of production yields rather than on finding new biochemical features in biocatalysts. This happens because the isolation of novel microbial enzymes is not an easy undertaking. Microbiologists estimate that only approx. 10% of all microorganisms in a given environment are actually cultivable (Breithaupt 2001). Several approaches have been followed in efforts to find novel enzymatic systems, some of which will be discussed in the next section.

Search for novel systems – classical and improved approaches

New technologies for manipulation, coupled with an increased understanding of fundamental biology and bioinformatics, have been shaping the discovery, purification and application of enzymes (Beilen & Li 2002). The first step is identification of a target reaction within an existing industrial process that can benefit from use of enzymes. An ideal biocatalyst is suggested and then actively sought, based on existing reaction constraints. For that purpose, biocatalyst screening or engineering (or a combination of both) is performed (Figure 1).

Biocatalyst screening

The genetic diversity of nature is still the major asset in terms of proteolytic enzymes to be used in textile and agro-industries (Burton et al. 2002). Many habitats warrant comprehensive bioprospecting, especially the microbial communities established in environments with extremes of temperature, salinity, pressure and pH (Niehaus et al. 1999; Breithaupt 2001; Demirjian et al. 2001; Burg 2003). Although thermophilic extremophiles have attracted most attention, psychrophiles are also interesting sources of industrial enzymes because of the ongoing efforts to decrease energy consumption worldwide (Burg 2003). Thermophilic peptidases have found application in detergent and controlled hydrolyses of food and feed, psychrophilic peptidases as detergent additives, halophilic peptidases in peptide synthesis, alkalophilic peptidases also in detergent formulation and acidophilic peptidases in feed formulation (Niehaus et al. 1999; Breithaupt 2001; Burg 2003; Fujinami & Fujisawa 2010).

Despite intensive research efforts to find and characterize new microbial sources of enzymes, only a small proportion of microbial species is currently cultivable, which restricts access to microbial genomes and gene products (Burton et al. 2002). Modern bioprospecting methods, for example, multiplex or metagenome cloning, have been increasingly employed to directly access environmental genomes, either culturable or not (Burton et al.

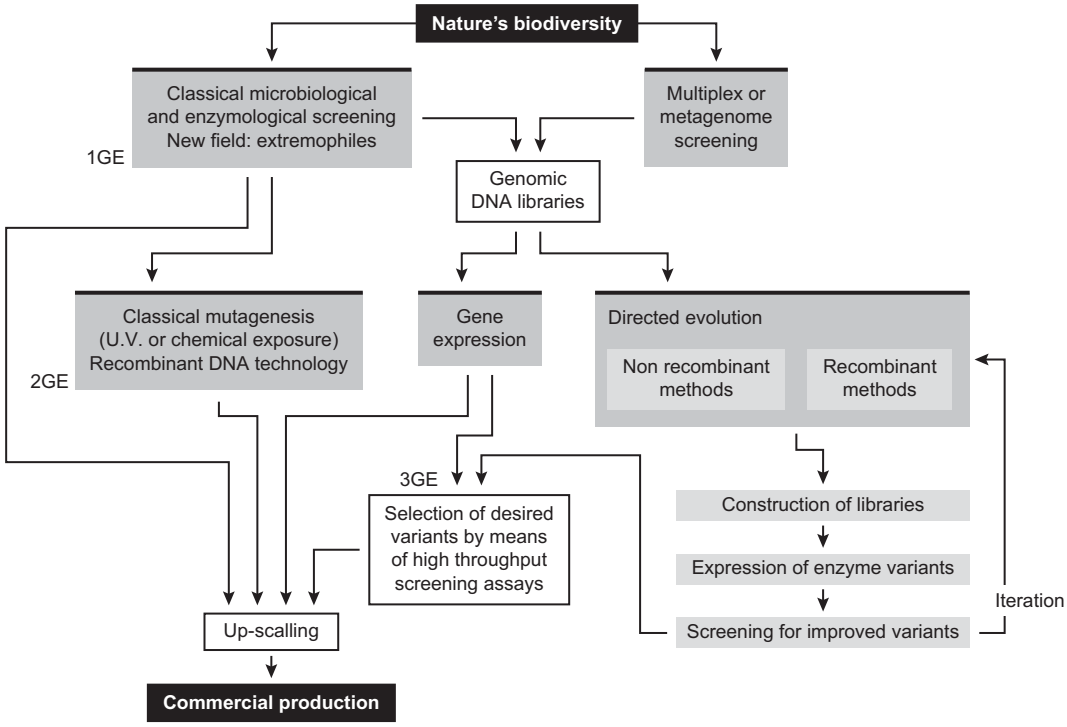

Figure 1. Schematic representation of classical and novel 'research-flows' leading to commercial protease production. 1GE, 2GE and 3GE correspond to first, second and third generation enzymes, respectively (adapted from Kirk et al. 2002).

2002), and constitute an alternative way to screen for biodiversity in nature. This demands high-throughput screening methods for biocatalysts (Wahler & Reymond 2001), to inexpensively screen genomic DNA libraries previously obtained either from biosphere sampling (through classical or genomic approaches) or from diversity generation methods (e.g. error-prone PCR, or gene shuffling of an existing enzyme gene or gene family). Recent developments in high-throughput enzyme assays have included analytical instruments for parallel screening, thermistor arrays and new fluorogenic and chromogenic substrates, as well as novel applications of pH indicator methods (Wahler & Reymond 2001). High-throughput screening approaches incur a significant cost, but this will reduce as robotics become more and more widely available.

Directed enzyme evolution techniques (see Figure 1) have emerged as a tool to generate enzymes with unconventional, improved features and tailored to production processes; this has been demonstrated in academic and industrial settings (Arnold & Volkov 1999).

Biocatalyst engineering

Although search for novel proteolytic enzymes has been successful, several methodologies are available to improve existing ones (Breithaupt 2001). Enzymes found in nature are often not readily available in quantities sufficient for industrial use, so use of homo- or heterologous gene expression systems to express recombinant proteins is required (Araújo et al. 2008). In addition, protein engineering based on random or site-directed mutagenesis (Gupta et al. 2002b) can give relatively rapid and inexpensive incremental improvements in selected enzyme features, with a reduced risk of losing desirable enzyme characteristics along the process (Burton et al. 2002). Furthermore, rapid developments in DNA sequencing will permit the genomes of many more industrially relevant microorganisms to be completely resolved in the near future (Beilen & Li 2002).

Despite the recent development of sophisticated molecular engineering and screening technologies, the ability to move around sequence space in search of the ideal biocatalyst for a given process is still limited (Burton et al. 2002). New technologies for biocatalyst screening and engineering will outcompete classical ones, yet the combined use of rational protein engineering, directed evolution and nature's own biodiversity will be far superior to any stand-alone technology (Kirk et al. 2002).

Peptidase assays

Textile and agro-industrial applications impose specific restrictions upon the desirable features of proteases, including robust, selective and highly

active enzymes (Beynon & Bond 2001; Gupta et al. 2002a). Measurement of the products of protein hydrolysis or of residual protein itself constitutes the basis of any attempt to assay for peptidases (Gupta et al. 2002a). Quantification of proteolytic activity then depends on the nature of the substrate and the sensitivity and precision needed (Beynon & Bond 2001). Both natural and synthetic substrates can be used for relevant peptidase assays, but a few constraints may apply depending on the nature of the enzyme.

Natural substrates, viz. gelatin, casein and hemoglobin, are commonly used in endopeptidase assays, but are not routinely used for assaying exopeptidases (Beynon & Bond 2001). One of the greatest problems in assaying endopeptidases is indeed appropriate choice of substrate. The use of synthetic substrates is generally not recommended, so development of a suitable assay is more complex. However, if the nature of the enzyme is already suspected (e.g. based on bioinformatics) then a natural substrate best suited for the assay is usually available, for example, casein for caseinase, keratin for keratinase or gelatin for gelatinase (Beynon & Bond 2001).

Several methods have been proposed in the literature to assay for peptidase activity; an overview of the most relevant qualitative, semi-quantitative and quantitative methods in routine use is given in Tables III and IV.

Qualitative and semi-quantitative methods

In academic research on peptidases, qualitative methods are commonly employed in initial screening. Most assays resort to solid-phase matrices on which either the enzyme or the substrate are immobilized; this is the case of electrophoresis and plate assays (Beynon & Bond 2001).

Screening procedures based on agar plate assays, in which enzymes diffuse into a gel matrix containing a hydrolysable substrate, have been in use for a long time, mainly for detection of extracellular proteolytic activity in microorganisms (Frazier & Rupp 1928; Šafařík & Šafaříková 1994). However, detection is sometimes difficult due to the low contrast between un-hydrolyzed and hydrolyzed areas on the agar plate. Therefore, overlay of a suitable precipitating agent is recommended to improve resolution, even though some damage may result upon the colonies (Šafařík & Šafaříková 1994; Saran et al. 2007b). When the aim of a given study is not only to find protease producers but also to isolate them for *a posteriori* studies, use of a precipitating agent may be limited.

Many protein substrates have been employed in agar plate assays, viz. skimmed milk, casein, calcium caseinate and feathers (Kanekar et al. 2002; Queiroga et al. 2007; Tatineni et al. 2008; Zhang et al. 2009), but the underlying principle is the same, that is, creation of a clearance zone as a result of enzyme-mediated substrate hydrolysis (Gupta et al. 2002a). Several authors have used skimmed milk agar to screen for protease producers (Table III); one disadvantage is that acid-forming bacteria can produce clearance zones on such a medium that are not a result of proteolysis (Martley et al. 1970). Consequently, the use of distinct protein agar formulations, for example, calcium caseinate agar, has been suggested (Queiroga et al. 2007) because this allows good visualization of the digestion halos, while their opaque regions are acid tolerant. A similar solid-phase qualitative (and even semi-quantitative) method is the radial diffusion assay, whereby protease is detected via observation of the hydrolysis zone building around small wells cut in agar plates containing immobilized substrate (Gupta et al. 2002a).

Besides radial diffusion and protein agar plate assays, zymography is a semi-quantitative solid-phase technique that has been extensively used in attempts to detect proteolytic activity, owing to its simplicity and sensitivity (Quesada et al. 1996). Zymograms

Table III. Overview of qualitative and semi-quantitative assays for proteases reported in textile- and agro-industrial-related literature.

Method	Substrate	References
	Calcium caseinate	Queiroga et al. (2007)
Protein agar plate	Casein	Tatineni et al. (2008)
	Feather	Mabrouk (2008); Zhang et al. (2009)
Radial diffusion assays	Skim milk	Mehrotra et al. (1999); Kanekar et al. (2002); Nadeem et al. (2007); Saran et al. (2007b); Devi et al. (2008); Tang et al. (2008); Abusham et al. (2009); Fang et al. (2009); Mala and Srividya (2010)
Liquid medium	Intact feathers	Tatineni et al. (2008); Xu et al. (2009); Jaouadi et al. (2010)
	Azocasein	Reddy at al. (2008a)
	Casein	Prakash et al. (2005); Tang et al. (2008); Moradian et al. (2009)
	Feather meal	Riessen and Antranikian (2001)
Zymography	Gelatin	Prakash et al. (2005); Kim et al. (2007); Tatineni et al. (2008); Chen et al. (2011)
	Keratin	Kainoor and Naik (2010)
	Skim milk	Kim et al. (2007)

allow identification of a protease of interest within a complex mixture of enzymes. It is essentially a gel electrophoresis method where the substrate is incorporated in a gel matrix; protease-containing zones will show up as areas where the proteinaceous substrate has been depleted from the gel. Several proteinaceous substrates have proven adequate for zymography, although gelatin and casein are used most frequently (see Table III). Proteases that possess the ability to renature and exert proteolytic activity on a copolymerized substrate, upon removal of SDS, can be analyzed by this method (Leber & Balkwill 1997). While other qualitative and semi-quantitative methods are used almost exclusively for screening purposes, zymography has been often employed when a certain degree of purification had been previously attained to rapidly assess the presence and level of activity of proteases in the purified extract.

Quantitative methods

Methods aimed at quantifying proteolytic activity actually measure the extent of proteolysis, and in general consist of liquid-phase assays: spectrophotometry, fluorimetry, radiometry, chromatography (HPLC), capillary electrophoresis and enzyme-linked immunosorbent assays (ELISA) are commonly used, with natural or synthetic substrates. Several novel assays can be found in the literature, yet most research programs still persist in using older procedures for assay of proteases (Beynon & Bond 2001).

Spectrophotometric techniques are probably the most widely accepted methods of assay for proteolytic activity, relying on differences in molar absorptivity between substrate(s) and product(s). A range of spectrophotometric assay conditions for peptidases of interest to textile and agro-industrial-related endeavors is suggested in Table IV. For measuring keratinase activity, keratin azure is widely accepted as substrate, and the most suited wavelength is 595 nm (al-Sane et al. 2002; Cai et al. 2008; Mabrouk 2008; Tatineni et al. 2008; Jaouadi et al. 2009; Syed et al. 2009; Elíades et al. 2010).

Production of proteases

One of the major constraints in the industrial application of enzymes (and other metabolites, for that matter) from microbial sources is the low productivity typical of fermentation processes. To enhance the production of proteases, concerted

Table IV. Overview of peptidase spectrophotometric assay conditions reported in textile- and agro-industrial-related literature.

	Assay conditions (ranges)			
Substrate	Temp (°C)	Wavelength (nm)	Time	References
Azocasein	25–55	340–450	6 min–3 h	Manczinger et al. (2003); Moreira et al. (2003); Najafi et al. (2006); Olivera et al. (2006); Thys et al. (2006); Dienes et al. (2007); Meza et al. (2007); Chen and Wang (2008); Abusham et al. (2009); Infante et al. (2010); Chen et al. (2011)
Azokeratin	37–60	440–450	15 min–30 min	Riffel et al. (2003a); Xu et al. (2009); Zhang et al. (2009); Chen et al. (2011)
Casein	30–60	280–660	10 min–1 h	Kamal et al. (1995); Garcia-Kirchner et al. (1998); Matta and Punj (1998); Mehrotra et al. (1999); Riessen and Antranikian (2001); Geok et al. (2003); Banik and Prakash (2004); Gupta et al. (2005); Li et al. (2005); Prakash et al. (2005); Rahman et al. (2005); Genckal and Tari (2006); Tari et al. (2006); Zhang et al. (2006); Anandan et al. (2007); Kim et al. (2007); Nadeem et al. (2007); Queiroga et al. (2007); Saran et al. (2007a); Wang et al. (2007); Huang et al. (2008); Reddy et al. (2008a); Tang et al. (2008); Vonothini et al. (2008); Wang et al. (2008a, b); Jaouadi et al. (2009); Moradian et al. (2009); Syed et al. (2009); Zhou et al. (2009); Jaouadi et al. (2010); Mala and Srividya (2010); Manni et al. (2010); Romsomsa et al. (2010)
Keratin	37–75	280–660	10 min–1 h	Garcia-Kirchner et al. (1998); Cai and Zheng (2009); Kainoor and Naik (2010)
Keratin azure	28–50	595	30 min–24 h	al-Sane et al. (2002); Cai et al. (2008); Mabrouk (2008); Tatineni et al. (2008); Jaouadi et al. (2009); Syed et al. (2009); Elíades et al. (2010)
Other substrates	37–75	245–595	15 min–1 h	al-Sane et al. (2002); Manczinger et al. (2003); Riffel et al. (2003a, b); Anbu et al. (2005); Najafi et al. (2006); Anandan et al. (2007); Jaouadi et al. (2009, 2010); Vishwanatha et al. (2010)

efforts have focused on the physiology of the microorganisms or on the design of the bioreactors and bioprocesses (Burton et al. 2002). The key approaches encompass continuous fermentation bioprocesses, specific medium, innovative bioreactor implementation and overproduction in mesophilic hosts (Schiraldi & De Rosa 2002). It is beyond the scope of this review to explore in detail the latter two approaches, so an emphasis will be placed on improvement of bioprocesses and medium engineering.

Fermentation processes

Several commercially available enzymes are relatively inexpensive, but many interesting ones are typically too costly for wide application (Beilen & Li 2002). Since reduced production costs can considerably expand the range and intensity of their applications as catalysts, efforts have been devoted to develop alternative technologies for peptidase production, especially solid-state fermentation (SSF) (Lazim et al. 2009; Rai et al. 2009; Vishwanatha et al. 2010).

SSF involves growth of microorganisms on moist solid substrate(s), in the absence of free-flowing water (Mukherjee et al. 2008). Peptidases are generally produced by submerged fermentation (SmF) (Gupta et al. 2002a), but the use of SSF brings a number of advantages like lower production costs (lower water and energy requirements), less waste generation and increased product stability (Mukherjee et al. 2008; Lazim et al. 2009). This type of fermentation is still not broadly implemented on a commercial scale, but advances in SSF technology are expected.

Medium design

Peptidase production from bacterial sources is usually constitutive or partially inducible (Gupta et al. 2002b), and controlled by various complex mechanisms that operate during transition between exponential growth and stationary phases, thus implying a strong dependence on medium ingredients (Gupta et al. 2002a), especially nitrogen and carbon sources. Optimization of the culture medium involves a large number of physiological and nutritional parameters, so medium composition needs to be determined on a case-by-case basis (Brandelli et al. 2010). The presence of easily metabolizable sugars and divalent metal ions is crucial, coupled with optimal processing parameters, for example, pH, temperature, degree of aeration, density of inoculum and rate of stirring (Kumar & Takagi 1999). Optimization of processing parameters including medium components by changing 'one-variable-at-a-time' are extremely time-consuming and expensive when a large number of variables are to be tested (Rao et al. 2007). This approach also ignores interactions among parameters, yet it is still frequently used in bioprocess engineering (see Table V). This approach is useful mainly at the early stages of process optimization, when little is known about the factors that affect enzyme synthesis yield and rate.

The latest decade has witnessed increased use of statistical design methods to overcome the aforementioned inability to pinpoint interactions among processing parameters (Table V). Such improved screening methods include fractional factorial and Plackett–Burman designs, which are often used in screening for key response factors when more than three parameters are involved, and usually assume a suitable fractional experimental form to prevent an excessively large number of experimental runs (Myers & Montgomery 2002).

Response surface methodologies (RSM) are more suitable in the final steps of medium optimization. In essence, they are a collection of statistical and mathematical optimization techniques, which have accordingly experienced extensive applications in industry (Myers & Montgomery 2002). RSM allows experimenters to build polynomial models that can approximate the true response function within the vicinity of the true optimum (Puri et al. 2002). A number of selected examples of RSM applied for optimization of fermentation media are listed also in Table V.

Purification of proteases

Peptidases generally employed to commercial levels in the textile and agro-industries are crude extracts (Kumar & Takagi 1999), so purification is needed to better know the operational features of those enzymes. There are no strict rules for purification of peptidases, but a general scheme for purification can be outlined: product recovery, isolation and purification, and eventual stabilization (Gupta et al. 2002a).

An ideal process of enzyme recovery involves a small number of downstream steps (Burton et al. 2002). Typically this involves removal of cells, solids and colloids from the culture medium, usually by filtration or centrifugation (Kumar & Takagi 1999). The large volume of water from which the product has to be removed creates the need for concentration steps, for example, ultrafiltration, before purification (Gupta et al. 2002a). To remove bulk protein and prepare the extract for subsequent chromatography, salting out by solid ammonium sulphate,

Table V. Approaches over latest 5 years for improvement of protease and keratinase activity.

Approach	Methodology	Design variables	Organism (genera)	Yield gain	Software	References
Statistical	Placket–Burman RSM: CCD	(13) (4)	*Streptomyces*	4.96	Design Expert	Singh and Chhatpar (2010)
Statistical	Placket–Burman steepest ascent RSM: Box-Behnken	(10) (3) (3)	*Bacillus*	2.54	Design Expert	Liu et al. (2010)
Statistical	Placket–Burman RSM: CCD	(7) (3)	*Bacillus*	2.6	SPSS	Romsomsa et al. (2010)
Single-parameter		(20)	*Aspergillus*	n.a.		Kamath et al. (2010)
1-at-a-time		(19)	*Pseudomonas*	n.a.		Kalaiarasi and Sunitha (2009)
1-at-a-time	RSM: Box–Behnken	(29) (4)	*Aspergillus*	n.a.	Design expert	Vishwanatha et al. (2010)
Statistical						
1-at-a-time		(18)	*Penicillium*			Sindhu et al. (2009)
1-at-a-time		(7)	*Bacillus*			ul-Qadar et al. (2009)
Statistical	Placket–Burman RSM: CCD	(11) (4)	*Serratia*	n.a.	Design expert	Venil and Lakshmanaperumalsamy (2009)
1-at-a-time		(4)	*Bacillus*			Abusham et al. (2009)
Statistical	Placket–Burman RSM: CCD	(4) (3)	*Laccocephalum*	8.14	SAS Matlab	Zhou et al. (2009)
1-at-a-time		(29)	*Streptomyces*	n.a.		Lazim et al. (2009)
Statistical	RSM: 2^2 factorial with 3 centre points	(2)	*Aspergillus* (commercial)	n.a.	Statistica	Peričin et al. (2009)
1-at-a-time		(34)	Halophilic bacterium	n.a.		Joshi et al. (2008)
Statistical	Placket–Burman RSM: CCD	(13) (4)	*Halobacterium*	3.9	Design expert	Akolkar et al. (2009)
Statistical	RSM: Box–Behnken	(5)	*Shewanella*	n.a.	Design expert	Anbu et al. (2009)
1-at-a-time		(25)	*Halobacillus*	n.a.		Karbalaei-Heidari et al. (2009)
1-at-a-time		(6)	*Bacillus*	n.a.		Nadeem et al. (2008)
1-at-a-time		(1)	*Bacillus*	1.9		Mukhtar and Ikram Ul (2007)
1-at-a-time		(15)	*Streptomyces*	n.a.		Vonothini et al. (2008)
Statistical	Genetic algoritm and particle swarm optimization		*Bacillus*	n.a.	Matlab	Skolpap et al. (2008)
1-at-a-time		(3)	*Roseobacter*	n.a.		Shanmughapriya et al. (2008)
1-at-a-time	Placket–Burman RSM	(14) (11) (3)	*Aspergillus*	14.0	Design expert SPSS Matlab	
Statistical						Hajji et al. (2008)
Statistical	RSM: CRFD	(3)	Commercial	n.a.	Statistica	Bhaskar and Mahendrakar (2008)
1-at-a-time		(25)	*Chryseobacterium*	n.a.		Wang et al. (2008b)
Statistical	RSM: CCD	(5)	*Bacillus*	>2.5	Matlab feed-fwd network	Subba Rao et al. (2008)
Statistical	RSM: CRFD	(4)	Commercial	n.a.	Statistica	Bhaskar et al. (2008)
Statistical	RSM: FCCCD	(5)	*Bacillus*	1.5	Design expert	Saran et al. (2007a)
1-at-a-time		(54)	*Aspergillus*	n.a.		Anandan et al. (2007)
1-at-a-time		(22)	*Chromohalobacter*	n.a.		Vidyasagar et al. (2007)
1-at-a-time		(10)	*Bacillus*	n.a.		Chu (2007)
1-at-a-time	Placket–Burman RSM	(19) (10) (3)	*Bacillus*	6.25	Design expert	
Statistical						Tiwary and Gupta (2010)
1-at-a-time		(21)	*Bacillus*	n.a.		Kumar et al. (2010)
Statistical	RSM: CCD	(5)	*Streptomyces*	3.5	Statistica	Tatineni et al. (2007)
	RSM: CCD	(3)				

Statistical	RSM: CCD	(3)	*Bacillus*	3.0	Design Expert	Radha and Gunasekaran (2007)
Statistical	RSM: Box–Behnken	(7)	*Scopulariopsis*	n.a.	Design Expert	Anbu et al. (2007)
Statistical	RSM	(3)	*Bacillus*	n.a.	Design Expert Statistica	Ladeira et al. (2010)
1-at-a-time Statistical	RSM	(14) (3)	*Bacillus*	n.a.	Matlab Statistical softw.	Rai and Mukherjee (2010)
1-at-a-time Statistical	RSM: CCD	(15) (4)	*Bacillus*	n.a.	Statistica	Huang et al. (2008)
1-at-a-time		(5)	*Bacillus*	n.a.		Nascimento et al. (2007)
Statistical	RSM: CCD	(3)	*Bacillus*	6.0	Statistical pack.	Oskouie et al. (2008)
Statistical	Placket–Burman RSM: CCD	(8) (3)	*Bacillus*	2.3	Design Expert	Reddy et al. (2008b)
1-at-a-time Statistical	RSM: 2^4 CCD	(24) (4)	*Colwellia*	n.a.	Design Expert	Wang et al. (2008a)
1-at-a-time Statistical	FFD RSM: CCD	(14) (6) (2)	*Bacillus*	1.7	Design Expert	Cai and Zheng (2009)

or use solvent extraction using acetone and ethanol are often employed (Beynon & Bond 2001; Gupta et al. 2002a).

Following initial fractionation, the peptidase is either re-suspended in, or dialyzed against the buffer to be used in the next step. To further purify the enzyme, a combination of column chromatography techniques is the usual routine (Gupta et al. 2002a). Commonly used chromatographic techniques include affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography, gel filtration and FPLC (Kumar & Takagi 1999; Gupta et al. 2002a). Ion exchange chromatography typically precedes size exclusion chromatography because of the limitation of sample size in the latter (Beynon & Bond 2001). A few dedicated techniques for proteolytic enzymes, for example, cysteine-type, trypsin-like and metallo-endopeptidases, have been described as well (Beynon & Bond 2001), but it is crucial to carefully inspect all steps of the purification protocol in order to promptly pinpoint less efficient ones and thus conveniently design strategies for improvement.

Industrial impact of enzyme technology

Enzyme technology is a truly interdisciplinary field and widely recognized as an important component towards white biotechnology. In agriculture, use of biocatalysts as feed additives has positive effects upon environment, animal health and metabolic efficiency. For cleaning, enzymes are used as ingredients of detergents thus increasing stain removal at competitive prices. Enzymes from extremophiles are used in paper and textile applications as well, with a positive environmental impact (Beilen & Li 2002).

Textile processing and agro-industrial applications

Textile processing and, more recently, agro-industrial processing have benefited greatly from the use of proteolytic enzymes, in terms of both environmental impact and product quality (Kumar & Takagi 1999; Araújo et al. 2008). Peptidases remain the dominant hydrolytic type of enzymes in the industry due to their extensive use in detergent and dairy industries (Kirk et al. 2002).

A number of reviews have discussed the applications of proteolytic enzymes in these fields (Anwar & Saleemuddin 1998; Rao et al. 1998; Horikoshi 1999; Kumar & Takagi 1999; Gupta et al. 2002b; Kirk et al. 2002; Haki & Rakshit 2003; Maurer 2004; Saeki et al. 2007; Araújo et al. 2008; Kumar et al. 2008). Recently, some authors have devoted their attention specifically to the applications of keratinases (Beilen

& Li 2002; Gupta & Ramnani 2006; Brandelli 2008; Brandelli et al. 2010), so a brief overview is presented below.

Protease-based wool finishing and hide-dehairing

Wool is a complex proteinaceous matrix, the surface scales of which account for the distinctive felting and shrinking properties upon wet-processing. Since consumers prefer machine washability, the market value of wool has steadily decreased. Applications of enzymes to wool may bring added value since wool fibers consist mainly of proteins and lipids. Proteases and lipases are therefore new promising processing routes (Queiroga et al. 2007).

The potential of proteolytic enzymes has recently been assessed with regard to removal of wool fiber scales for improvement of the anti-felting behaviour of wool (Montazer & Ramin 2010; Raja & Thilagavathi 2010; Cai et al. 2011). However, an enzyme-based industrial process for finishing has not yet been established. This is mainly due to technological difficulties in controlling fibre damage by proteases. Proteolytic enzymes are able to penetrate the fibre cortex due to their small size, thus compromising the inner parts of the wool structure (Araújo et al. 2008). Efforts have been made to increase the size of the enzyme to reduce penetration, by chemical cross-linking or attaching synthetic polymers (Schroeder et al. 2004, 2006; Silva et al. 2004).

Alkaline proteases, and especially keratinases, without collagenolytic activity but with mild elastolytic activities, offer the possibility of an effective biotreatment of leather, particularly in terms of dehairing and bating of skins and hides, as a suitable alternative to conventional tannery processes that resort to sulfide (Brandelli et al. 2010). In the traditional process, hair is gelatinized and converted into a pulp, whereas hair remains intact in the enzymatic process. Proteolytic enzymes able to meet these specifications should help break down the keratin tissue in the follicle, thereby removing intact hair without affecting the tensile strength of the leather (Gupta & Ramnani 2006). This would result in production of higher-quality leather and would also lead to improvement in wastewater quality, thus reducing pollution. A number of studies have been successful in using proteolytic enzymes for leather tanning (Riffel et al. 2003b; Macedo et al. 2005; Giongo et al. 2007; Jaouadi et al. 2009).

Protease-based laundry detergents

The use of proteases as detergent additives still represents the largest single use of industrial enzymes (Kirk et al. 2002). Over several years, subtilisins have been established as suitable detergent proteases because they efficiently hydrolyse insoluble protein-based stains in alkaline thermophilic environments and several improvements have resulted from use of various forms of enzyme technology (Maurer 2004). Alkaline thermophilic microorganisms are preferred sources of proteolytic enzymes for this purpose, since their thermophilic enzymes are claimed to hasten the hydrolysis process and diminish the risk of contamination, while withstanding harsh washing conditions (e.g. agitation, and presence of surfactants and oxidizing agents). However, processes using mesophilic ones are less energy-consuming (Brandelli et al. 2010).

In the future, the detergent market is expected to redirect toward cooler washing steps that will decrease the efficiency of traditional ingredients, so enzymes from psychrophilic microorganisms may be in demand for detergent formulation (Burg 2003). Extensive research has been conducted on the use of alkaline thermostable proteases; however, cold-active enzymes are very poorly studied despite the market needs for cold washing processes (Wang et al. 2008a; Yang et al. 2010).

Protease-based processing of keratin-rich wastes

Microbial keratinases have attracted a great deal of attention in the past decade, due to their ability to improve several industrial processes. When compared to other proteolytic enzymes, industrial applications of keratinases are limited, but they have found use in processing keratin residues, for example, production of feed hydrolysates, feed supplements and nitrogen fertilizers (Brandelli et al. 2010).

Of particular interest are spent feathers, which can be converted to feather meal under high temperature and pressure and then used as animal feed supplement (Gupta & Ramnani 2006). However, this is an expensive approach and their poor digestibility and low nutritional value have suggested the use of keratinases to pre-hydrolyze feather into a better nutritional ingredient (Onifade et al. 1998). The protein-rich hydrolysate generated from poultry can be useful in the preparation of nitrogen fertilizers or soil amendments (Brandelli 2008), and the potential conversion of keratinous wastes into biodegradable films and glues, for compostable packaging or edible film applications, has been reported as well (Gupta & Ramnani 2006).

Constraints on large-scale application of proteases

Despite extensive research, there are still numerous gaps in our understanding of proteases in relation to

their application to the textile and agro-industries (Rao et al. 1998). The stability of proteases remains a critical issue; both storage and operational stabilities affect the usefulness of enzymes as processing aids (O'Fagain 2003). Protein engineering, chemical modification and addition of stabilizing compounds are the main techniques employed at present for enzyme stabilization.

Immobilization may lead to enhanced stability gains, but it is generally undertaken to prevent loss of a biocatalyst or to improve bioreactor operation (O'Fagain 2003). Prevention of autoproteolytic inactivation, change of substrate specificity and improvement of yield are important issues to be addressed (Rao et al. 1998).

Final considerations

Many research studies have demonstrated the potential role of proteolytic enzymes in textile and agro-industrial applications. Nevertheless, commercial applications (and subsequent industrial market demand) for such enzymes are still in their infancy, chiefly because of scale-up and downstream processing constraints. Since proteases featuring unique physicochemical characteristics already play important roles in industry, research toward development of more robust proteases, especially keratinases, should be encouraged.

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