Review

An Overview of Current and Novel Approaches for Microbial Neutral Protease Improvement

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Abstract: Neutral proteases are of considerable interest in view of their activity and stability at pH of 6.0-7.5. This review describes the current and novel approaches for microbial neutral protease improvement and their potential application in diverse industries. Various hosts for expression are discussed which confer the screening and modification of the genes with high efficiency. We give a special focus on strain improvement using recombinant DNA technology and mutagenesis which can be applied to augment the efficiency of the producer strain to a commercial status. Further, medium formulation and fermentation technologies considering the nutritional and environmental parameters affecting the production are delineated.

Keywords: microbial neutral proteases; strain improvement; production.

1. Introduction

Proteases are classified into acid, neutral and alkaline protease on the basis of the optimum activity pH range, of which neutral and alkaline proteases possess the highest casein-hydrolyzing activity. Neutral proteases (NPRs) are mostly metallo-endoproteinases which show optimum activity while pH = 6.0-7.5. These enzymes contain one zinc atom per molecule which is essential for catalytic activity, and calcium is required to maintain the structural rigidity of the molecule. There also exist cysteine proteases and serine proteases which work at neutral pH (Kumar and Takagi, 1999). Their structures vary and the resistances to inactivation differ from each other markedly. Many properties of
NPRs are quite distinct from the alkaline proteases isolated from various strains of *B. subtilis* (McConn *et al.*, 1964).

It is well known that proteases constitute one of the most important industrial enzymes, and NPR was the first discovered and have a large variety of industrial application, such as food, pharmaceutical, cosmetic, *etc.* and have been widely commercialized by various companies throughout the world. A number of publications on the synthesis of exoenzymes have appeared since 1969 (Schaeffer, 1969). Among all the microbes, *Aspergillus* and *Bacillus* have many advantages for NPRs production, since they are normally GRAS (generally regarded as safe) strains and the produced enzymes are extracellular, which make it easy recuperation from fermentation broth. In addition, there are many possibilities for their improvement. In 1983 the first cloning and expression of the structural gene from *B. stearothermophilus* in *B. subtilis* was reported (Fujii *et al.*, 1983) and the period of gene modification on NPR improvement has begun since then. In the last few decades, great attention was attracted by the improving strategies in production processes including fermentation processes, medium design, and purification. After all, construction for strains of high protease activity is still an important issue all around the world.

In the present review, some aspects of the microbial NPRs are discussed, with reference to their industrial application, along with the upcoming approaches for developing novel proteases with higher productivity, activity and stability using new technologies.

**2. Sources and Application of NPRs**

**2.1. Sources and Discovery**

Table 1 describes the cloning and expression of genes for several well recognized NPRs from microorganisms. It is indicated that *Bacillus* is the most important source of NPR genes. According to the classification of proteases based on protein structure and homology implemented in the MEROPS database (http://merops.sanger.ac.uk), a cluster of NPRs in reports could be classified as neutral protease E, which is recommended naming as bacillolysin, including *Bacillus subtilis* neutral proteinase, Bae16 peptidase, NprA g.p., NprE g.p., NprM g.p., NprS g.p., and thermolysin homologue. Neutral protease B from *Bacillus sp.* shares a relatively low homology with the proteases above (Tran *et al.*, 1991). *B. stearothermophilus* produces a thermostable protease, thermolysin (Takagi *et al.*, 1985). *Clostridium histolyticum* and *Streptococcus* spp. produce clostripain and streptopain, respectively, which are cysteine proteases. *Pseudomonas aeruginosa* and *Streptomyces griseus* are also neutral metalloprotease producers (Morihara *et al.*, 1968). *A. oryzae* is one of the most important fungal NPR producers (Zhao *et al.*, 2012). A few more species have been reported to produce NPRs such as *A. sojae* (SEKINE, 1972), *Penicillium* (Germano *et al.*, 2003), *Ustilago maydis* (Hellmich and Schauz, 1988), *Rhizopus* (Haq and Mukhtar, 2004), *Microbacterium* (Thys *et al.*, 2006), *Streptomyces*.
from Actinomycetes (Butler et al., 1992), etc.

In spite of the availability of large number of NPRs, robust proteases which require little or no reengineering for industrial applications is yet to be identified. The novel proteases should be better suited to the various industrial processes/products, including substrate conversion, narrow/broad substrate specificities, stability, and activity, than the currently available enzymes (Kasana et al., 2011). Besides, proteases with new specificities and stability under extreme conditions are required for various applications. Hence, exploring the new niches for new proteases is of great significance.

Table 1. Microbial sources of neutral protease or cloning, sequencing, and expression of the neutral protease genes

<table>
<thead>
<tr>
<th>Source</th>
<th>Characterization of gene</th>
<th>Host &amp; usage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>nprE</td>
<td>create of an in vitro-derived deletion mutation</td>
<td>Yang et al., 1984</td>
</tr>
<tr>
<td></td>
<td>nprE</td>
<td>Saccharomyces cerevisiae</td>
<td>Wang and Devenish, 1993</td>
</tr>
<tr>
<td></td>
<td>nprE</td>
<td>Lactococcus lactis subsp</td>
<td>van de Guchte et al., 1990</td>
</tr>
<tr>
<td></td>
<td>nprB</td>
<td>B. subtilis WB30</td>
<td>Tran et al., 1991</td>
</tr>
<tr>
<td>B. subtilis, from factory</td>
<td>nprE</td>
<td>Pichia pastoris SMD1168</td>
<td>Xu et al., 2005</td>
</tr>
<tr>
<td>B. amyloliquefaciens F</td>
<td>- a</td>
<td>B. subtilis 1A289</td>
<td>Honjo et al., 1984</td>
</tr>
<tr>
<td>B. amyloliquefaciens ATCC 23844</td>
<td>npr[BamP]</td>
<td>B. subtilis BR151, IS2, IS4</td>
<td>Vasantha et al., 1984</td>
</tr>
<tr>
<td>B. stearothermophilus CU21</td>
<td>nprT, thermostable; highly homologous to that of the thermostable NPR from B. thermoproteolyticus but less homologous to that from B. subtilis</td>
<td>B. subtilis MT-2</td>
<td>Takagi et al., 1985</td>
</tr>
<tr>
<td>B. stearothermophilus Rokko</td>
<td>- a</td>
<td>E. coli, the propeptide is not required for activity</td>
<td>Mansfeld et al., 2005</td>
</tr>
<tr>
<td>B. thermoproteolyticus Rokko</td>
<td>npr, thermolysin, it has 100% sequence similarity with the</td>
<td>B. subtilis DB117, indistinguishable</td>
<td>O'Donohue et al., 1994</td>
</tr>
</tbody>
</table>
**B. thermoproteolyticus**  
*npr*, thermolysin  
*E. coli* K12, JM109, indistinguishable from natural thermolysin  
Inouye *et al.*, 2006

**B. nematocida**  
*npr* Bae16, virulent against nematodes; it has 94% sequence identity with *npr* gene from *B. amyloliquefaciens*, but has low similarity (13–43%) with the reported virulence serine proteases from fungi or bacteria  
*E. coli* BL21  
Niu *et al.*, 2006

**B. cereus**  
*cnp*, thermolysin-like protease  
*B. subtilis*  
Wetmore *et al.*, 1992

**B. caldolyticus**  
*npr*, highly similar to that of the *B. stearothermophilus npr* gene. They differed at only three amino acid positions  
*B. subtilis DB104, DB117*  
vander Burg *et al.*, 1991

**Other bacteria**

- **Staphylococcus hyicus**  
  *ShpI* gene, the protease has a low substrate specificity  
  *Staphylococcus carnosus*  
  Ayora and Götz, 1994

- **Streptomyces cacaoi**  
  *npr*  
  *Streptomyces lividans*  
  Chang *et al.*, 1990

- **Salinivibrio proteolyticus**  
  *SVP2* gene  
  *E. coli* BL21, optimized with response surface methodology  
  Beigi *et al.*, 2012

- **Serratia marcescens**  
  *npr*  
  *E. coli*  
  Braunagel and Benedik, 1990

- **Vibrio proteolyticus**  
  *nprV*  
  *E. coli*  
  David *et al.*, 1992

**Fungi**

- **Aspergillus oryayaze**  
  *Np II*  
  *Saccharomyces cerevisiae*  
  Tatsumi *et al.*, 1991

**Note:** - *, no data available.
Since the production of NPRs is greatly influenced by strains, nutritional, and physicochemical factors, sensitive and specific detection assay as well as screening medium are necessary for sources screening. Reports have reviewed the knowledge of various methods and protocols available for proteases screening, detection, and quantification (Kasana et al., 2011). General screening could be executed with skim milk agar and casein agar plates for zone of clearance directly in the appropriate pH. However, the isolated bacteria weren’t always positive when NPR was desired, while the successful screening probabilities of other enzymes like alkaline protease will be improved if the screening conditions are severer. On the selection of psychrotrophic proteases (Kasana, 2010) and halophilic proteases (Vidyasagar et al., 2006), there have been lots of successful cases with specific modification on the media or culturing conditions.

Functional metagenomics comprising the isolation of DNA from environmental samples without prior enrichment of individual microorganisms, the construction of libraries from the recovered DNA, and function-driven screening of the generated libraries has led to the identification and characterization of a variety of novel enzymes. In the past few years, Metagenomic Libraries were constructed to increase the diversity for bioactivator screening, such as the isolation of metalloproteases with a novel domain structure by construction and function-driven screening of metagenomic libraries (Waschkowitz et al., 2009).

2.2. Application

The favorable pH for different processes varies. Adjustment of pH prior and/or during hydrolysis, although is common, is not industrially desirable as the added acid/alkali result in unwanted inorganic mass (salt) that may result in undesirable effects and/or may be difficult to remove later in a process (Aspmo et al., 2005). Due to the awareness for energy conservation, it is desirable to use proteases that are active at lower temperatures and native pH. In this regard, NPRs look promising wherein natural (in situ) pH in food industrial is important for protein hydrolysates, and critically, NPR treatment is effective to reduce the bitterness (Sandhya et al., 2005). Some enzymes used in food industrial include Neutrase®, Esperase®, Protamex®, and Novozym® FM, which are commercially marketed by Novozymes, Denmark, and Umamizyme® by Amano Enzyme, USA. These microbial proteases have application in functional, nutritional and flavor properties improvement. For example, Neutrase® is used in alcohol production for improving yeast growth. Reports are also available on rice starch isolation without additional chemicals (Wang and Wang, 2004), fresh fish waste treatment (Bhaskar and Mahendrakar, 2008), milk protein modification, animal feeds preparation (Dean and Ward, 1991), and so on. The low thermal tolerance and sensibility to chelating agents are advantageous for controlling their reactivity with a low degree of hydrolysis (Thys et al., 2006).

The application of NPRs is always under development. Dispase is produced by B. polymyxa. Its mild proteolytic action makes it useful for cell isolation and tissue dissociation. It’s is marketed by
many companies such as Invitrogen Corp., USA, BD Biosciences, USA, etc. Collagenase, which hydrolyses native collagen, has been used for debridement of dermal ulcers, burns and the lysis of diseased vertebral disks. Collagenase coupled with other NPRs is effective for pancreatic digestion (Bucher et al., 2004). Besides, NPRs are promising in many other fields such as agroindustrial wastes treatment (Brandelli, 2008) and bioinsecticides preparation (Zouari and Jaoua, 1999). Since NPRs are usually the virulence factors of many pathogenic bacteria, parasites and viruses (Sabotič and Kos, 2012), it’s great significative to study pathogenicity for pharmacy development.

3. Host Selections

The first study on cloning of the structural gene for a NPR from *B. stearothermophilus* (Fujii et al., 1983) was published in 1983. After that, a considerable number of reports have been published on the isolation and manipulation of microbial NPR genes with the aim of (1) protease overproduction by the method of heterologous expression, gene dosage effect, pathway regulation, etc., (2) protein engineering to locate the active site residues and/or to alter the enzyme properties to suit its commercial applications. Proteases naturally occurring in other organisms or found in minute concentrations in exotic microorganisms may now be produced in large-scale fermentation processes by selecting host microorganisms which are easy to cultivate on cheap raw materials and producing a broth from which the enzyme is easy to be purified. Naturally occurring proteases with interesting properties may be insufficiently characterized or related to a toxin-producing or to an opportunistic pathogenic organism. All these uncertainty can now be avoided. Host microorganisms for NPRs are listed as following.

3.1. *Escherichia coli*

Expression of recombinant proteins in *E. coli* can be performed fast and conveniently at little expense, and it is still the most commonly used host for industrial production of pharmaceutical proteins. It would enable us to carry out easily structural and functional analysis by manipulating a number of mutants and conducting library screening. However, some metallo-neutral proteases have been reported to express in *E. coli* in the form of inclusion bodies (Mansfeld et al., 2005; Trachuk et al., 2005), so the denaturation and refolding processes are needed to recover the active enzymes. Because of the lack on a general recovering procedure, *E. coli* is less preferentially used than *Bacillus*, though few procedures have been reported (Mansfeld et al., 2005). In contrast, the active form of thermolysin has been secreted successfully by *E. coli* (Inouye et al., 2006).

The major extracellular proteins of *Bacillus* and other gram-positives were not found to contain disulfide bridges (Bolhuis et al., 1999). As we know, proper disulfide bond formation is one of the most important processes for the activity and stability of proteases. Nevertheless, the formation of
Disulfide bonds in *E. coli* is a fast and effective process, which is catalyzed by thiol-disulfide oxidoreductases (Thwaite et al., 2002). Its disulfide system could even be used for the intentional formation of insoluble aggregates or “inclusion bodies” (LaVallie et al., 1993; Collins-Racie et al., 1995). Hence, the inactivation of NPRs from *Bacillus* in *E. coli* is probably due to “over-stability”. As a matter of fact, arguments such as lytic effect of the *nprE* gene product on *E. coli* host cells (Wang et al., 1990) are far from being settled down because of the limited researches.

3.2. Bacillus

The expression of the *nprE* gene in *B. subtilis* is subject to a complex, and not yet fully understood, mechanism of regulation (van de Guchte et al., 1990). In another word, it’s promising to improve the productivity with this host. *Bacillus* spp. secretes mainly two major types of protease, NPRE and serine alkaline protease, and their activity accounts for 20% and 70%, respectively. *B. subtilis* secretes numerous enzymes to degrade a variety of substrates, enabling the bacterium to survive in uncomfortable environment and utilize cheap raw materials. These enzymes are produced commercially and their production represents about 60% of the industrial-enzyme market (Westers et al., 2004). Studies of these extracellular proteases are important for understanding the mechanism of secretion. Besides, *B. subtilis* is considered as a GRAS organism, which makes its usage highly favored over *E. coli*. The NPRE expression was found to be tightly associated with sporulation and affected by culturing conditions in early studies (Jansova et al., 1993). The RNPP protein family seems to participate in transcription regulation, and the structure of expression vector is critical in efficiency. These factors would be discussed in the following.

3.3. Lactococci

Many heterologous proteins have already been produced in *Lactococci*, especially the *L. lactis*, but only few of them allow comparing production yields for a given protein either produced intracellularly or secreted in the medium (Le Loir et al., 2005). In this system, it was indicated that secretion is preferable to cytoplasmic production, and secretion enhancement by signal peptide and propeptide optimization could result in increased production yield (van de Guchte et al., 1992; Hammes and Hertel, 2006). The complex control mechanisms of *Bacillus* could be bypassed by replacing the *nprE* promoter and preceding sequences with a lactococcal promoter in this bacterium. Because the additional NPR is often unevenly distributed in the cheese curd, the expression of NPR in *Lactococci* (*Lactococcus lactis* subsp. *lactis* and *cremoris*, previously *Streptococcus lactis* and *Streptococcus cremoris*, respectively) may be helpful in achieving accelerated cheese ripening (van de Guchte et al., 1990), the development of the texture and flavor of various dairy products, and making new applications possible (van de Guchte et al., 1992). Hence, the yield is not highlighted in this case.
The GRAS status of *Lactococci* and lactic acid bacterium in general, is a clear advantage for their use in production.

### 3.4. Other Hosts

Since the *npr* gene is plenty in the nature, host listed above are not enough. For example, the *npr* gene for neutral metalloprotease from *Streptomyces cacaoi* YM15 was expressed in *Streptomyces lividans* (Chang et al., 1990), the gene from *Staphylococcus hyicus* was expressed in *Staphylococcus carnosus*. *Aspergillus* is one of the main production strains for NPRs, but there’s no report for the usage as the host of heterogenous NPRs yet. A cDNA clone encoding *A. oryzae* NPRII was cloned and expressed in *Saccharomyces cerevisiae* (Tatsumi et al., 1991). However, the processing was affected easily by autolysis and yeast proteases degradation. The *nprE* gene was also cloned and expressed in *Saccharomyces cerevisiae* and *Pichia pastoris*, in which signal peptide is critical for secretion (Wang and Devenish, 1993; Xu et al., 2005). It seems to be improved in pH adaption and thermo-stability because of the slight glycosylation by yeast modifying the structure and function.

### 3.5. Construction of Strains with Reduced Protease Activity

Strains with reduced protease activity have been constructed in order to improve the production yield of heterologous proteins, and they provide an efficient tool for *npr* genes screening, because the proteases produced by the host microorganism is interferential for the judgment of heterologous or specific activities. *B. subtilis* strains with deletions in the *aprE* and *nprE* genes were first reported in the same issue of the Journal of Bacteriology in 1984 to produce very low extracellular protease activity. Since then, strains containing deletion mutations in multiple extracellular proteases have been constructed with extracellular protease activity of less than 0.5% compared to the parental strain (Sloma et al., 1991; Wu et al., 1991), and a strain WB800 deficient in 8 extracellular protease genes (*nprE, nprB, aprE, epr, mpr, bpf, vpr, wprA*) was obtained in 2002 (Wu et al., 2002). With the help of these systems, the emphasis is moving towards sourcing new genes of interest through database mining, unraveling the circuits related to gene regulation and applying, for example, transcriptomics. However, concerns regarding the use of these hosts were not upheld because their growth was reported to be limited (i.e., these strains would not always grow and sporulate normally) especially with industrial media (Schallmey et al., 2004).

### 4. Efficient Gene Constructions

Productivity of the natural microorganism has been improved by mutations, in some cases were up to a thousand fold, and the NPR production strains currently used are mainly from mutation. Since gene cloning is a rapidly progressing technology that has been instrumental in improving our
understanding of the structure-function relationship of genetic systems, it provides an excellent method for the manipulation and control of genes (Rao et al., 1998). Selection of an efficient gene construction may boost the NPR productivity in the post genome era. Heterogenous expression is restricted by many factors, in which the host characteristic and vector structure are basis and dementing (Klier et al., 1992). Current studies mainly focus on Bacillus expression system.

4.1. Promoter

In early study, the natural promoters of npr gene was replaced with the amylase promoter from B. amyloliquefaciens trying to increase the expression (Henner et al., 1985). As essential elements in expression, promoters contain specific DNA sequences and response elements that provide a secure initial binding site for RNA polymerase as well as transcription factors that recruit RNA polymerase. Various sites in a promoter domain play a role in promoting or suppressing expression of a gene by mediation of external signals, and that’s the mechanism of catabolite repression. Regarding the organizational form, Bacillus promoters could be classified into single promoters and chimeric promoters (Schumann, 2007). Most of the Bacillus single promoters are activated only at exponential phase, including the promoters of α-amylase, NPRE, and alkaline protease, while chimeric promoters, like overlapping promoter, function at exponential phase and stationary phase (Luo, 2007). In Bacillus, promoters with different degree of initiation efficiency have been isolated, and the strong gene promoters can be devised (Zhang et al., 2011). The P43 promoter is a well-characterized overlapping promoter that is functional during both the exponential and stationary growth phases (Wang and Doi, 1984), and it was successfully used for NPRE production with considerable yield. A strong promoter element PyxiE, yielding β-galactosidase twice as much as P43, was isolated in 2007 (Zhang et al., 2007).

Except for the strength and durability, a controllable expression system is desirable in efficient production of recombinant gene products. According to the intended type of control of gene expression, microbial promoters can be divided into constitutive promoters and chemical-inducible promoters. The latter are quite popular nowadays because the performance is artificially controlled with the compounds naturally not existed in the organisms. The promoter of sacB gene from its natural sucrose inducing system has been successfully used in over-production of NPR, α-amylase, etc. The most prominently and widely used induction systems in B. subtilis are mediated by promoters Pspac and Pxyl, which respond to the addition of IPTG and xylose, respectively (Härtl et al., 2001). But these systems use the costly inducer for industrial application. The recent developed maltose inducing promoter Pglv was quite strong and of acceptable cost (Ming et al., 2010). Amazing amounts of promoters that respond to antibiotics, copper, alcohol, steroids, and herbicides, among other compounds, have been adapted to allow the induction of gene activity at will (Goldstein and Doi, 1995). Along those lines, a strong and controllable system for NPR expression has broad prospect.
4.2. Signal Peptide

Secretion of the heterologous protein into the culture supernatant might be a limiting step for the yields of the exported proteins when optimized microorganisms with strong and inducible promoters, high-copy-number vectors, as well as improved codon are used. Extracellular NPRs are made with an amino-terminal extension, a signal peptide (SP), which targets the precursor to translocation sites on the appropriate membrane. During or shortly after translocation, SP is removed by a signal peptidase. Previous comparisons have shown that eukaryotic SPs differ in detail from prokaryotic ones (von Heijne, 1984; Nielsen et al., 1997), and that SPs from gram-positive bacteria tend to be longer than those from gram-negative species (Abrahmsen et al., 1985). Hence, SPs do not always function efficiently when expressing hosts are changed, which makes it important to predict and discover new SPs (Simon and Blobel, 1992; Dyrløv Bendtsen et al., 2004), e.g. the nprE gene has to be fused with yeast SP to express in Saccharomyces cerevisiae. To have better understanding of SP function in bacteria, which is primarily based on studies of E. coli, Bacillus SPs of alkaline and NPRs were altered on charged residues (Chen and Nagarajan, 1994). It was revealed that the charged residues at the N terminus are not essential for NPR SP function, while a mutant with a net charge of -1 was completely defective. Interestingly, introduction of a negatively charged residue at the N terminus of NPR SP blocked the export of fused levansucrase, indicating SP is even genetic and proteinic specific. The characterization of numerous extracellular proteins has allowed development of a computer program (SignalP 4.0) that uses artificial neural networks for prediction of the presence of SPs and for the location of SP cleavage sites (Petersen et al., 2011). The use of SignalP permits the design and preliminary analysis of SP derivatives prior to their construction and test in vivo.

4.3. Propeptide and Folding

Heterologous proteins that exhibit a slower folding in the cell wall microenvironment may be prone to degradation, and the folding of the proteins may need special attention (Jensen et al., 2000; Westers et al., 2004). Propeptides of NPR are stretches of amino acids located between the signal peptide and the mature part of the protein, which consists of around 194 residues (Yang et al., 1984). Similar to subtilisin, NPR is inactive without the propeptide, indicating that the propeptide is important for proper folding and maturation (Eder and Fersht, 1995). Besides, propepIdites prevent the activation of protease prior to their translocation (Wandersman, 1989). It could be provided in the form of either another propeptide molecule in trans or an added synthetic propeptide (Müller, 1992; Tjalsma et al., 2000). The propeptides may also indirectly affect the earlier steps of the export process, as indicated by two propeptide mutations of NPR that are deleterious for the host bacterium (Takagi and Imanaka, 1989). Analogously, mutations in the active sites of NPR prevent both enzymatic activity and processing, and the unprocessed precursors remain membrane associated but appear to be located on
the outer surface of the cytoplasmic membrane (Toma et al., 1989). It was suggested that a feedback mechanism between synthesis and export may function in the cells, because the mutations of active site reduced the amount of protein synthesized, which is probably caused by the uncleaved signal peptides occupying the export sites (Simonen and Palva, 1993).

In contrast, it is reported that the propeptide is not required to produce catalytically active NPR from \textit{B. stearothermophilus} (Mansfeld et al., 2005). According to the study, NPR was expressed in the form of inclusion bodies and renatured in high yields. A recombinant thermolysin was expressed actively by co-expressing the mature sequence and pro-sequence in \textit{E. coli} (Yasukawa et al., 2007). Hence, expressing the mature peptide may be an alternative method for NPR synthesis, especially in the host that folding could not process accurately.

4.4. Chaperones

Chaperones have an important function in protein export by preventing the preproteins from folding into translocation-incompetent conformations. Some of the propeptides could directly catalyze the folding reaction and function as intramolecular chaperones (Shinde and Inouye, 2000). It was demonstrated that the thermolysin prosequence acts as an intramolecular chaperone \textit{in vivo}, which open the way to structural studies of catalytic site mutants produced in large quantities in \textit{E. coli} (Marie-Claire et al., 1999). Further, propeptide release, not precursor folding, is the rate-determining step in protein secretion (Yabuta et al., 2001), which could be explained with intramolecular chaperone mechanism. Similar to \textit{E. coli}, \textit{B. subtilis} has the GroE and the DnaK series of intracellular molecular chaperones. The genes for these chaperones are organized in two operons, the \textit{groE} operon (\textit{groES} – \textit{groEL}) and the \textit{dnaK} operon (\textit{hrcA- grpE- dnaK- dnaJ- yqeT- yqeU- yqeV}) (Li and Wong, 1992; Homuth et al., 1997), both of which are regulated by the repressor HrcA. It has been proved that the inactivation of \textit{hrcA} could increase the production of extracellular protease (Wu et al., 2002). The lipoprotein PrsA is an extracytoplasmic molecular chaperone (Jacobs et al., 1993). Its overproduction could also contribute to the expression improvement.

4.5. Genes of Quorum-sensing

Quorum-sensing (QS) is a bacterial mechanism for regulation of gene expression in response to cell density. The RNPP protein family (Rap, NP RR, PlcR, and PrgX) are intracellular QS receptors that bind directly to their specific signaling peptide for regulating the transcription of several genes in \textit{Bacillus} (Rocha et al., 2012). NP RR (neutral protease regulator) is an activator of NPR (Uehara et al., 1974 & 1979), and it is related to sporulation and extracellular protease activity. NPRRB is a small protein that includes the mature signaling peptide of NP RR (Pottathil and Lazazzera, 2003). The role of NPRRB as a signaling peptide hasn’t been well considered in early studies. Referring to PlcR,
NPRR and NPRRB are probably co-evolved, and the complex protein/peptide binds to DNA to activate the target genes (Rocha et al., 2012). It was suggested that synthetic peptides added in cultures can interact with NPRR in the intracellular space, and has the same regulation as NPRR. As described in previous studies, nprR occurs in two genotypes, nprR1 and nprR2, and organisms carrying nprR2 produce dozens of times more NPRE than the strains carrying nprR1 (Uehara et al., 1979; Toma et al., 1986). Excitingly, nprR is different from other regulation genes like sacU+, catA, which stimulate the production of NPR and alkaline protease simultaneously.

4.6. Other Factors on Regulation

It has been proved that the sequences of NPR vary a lot with different sources (B. stearothermophilus, B. thermoproteolyticus, B. subtilis, and B. amyloliquefaciens) (Takagi et al., 1985), especially for genes among genus. The differences among their regulation mechanism in transcription, translation and secretion may need special attentions. On the aspect of B. subtilis, except for the data above, there're a few reports on NPR production enhancement by overexpression of certain gene such as prtR (Yang et al., 1987), iep (Tanaka and Kawata, 1988), and by inactive mutation such as hpr locus (Perego and Hoch, 1988). These genes belong to deg (regulatory genes for degradative enzymes) family (Pang et al., 1991), of which the DegS/DegU two-component system contributes to the signal transduction network for bacterial stress responses (Mäder et al., 2002). However, the physical and chemical nature of signals that affect these regulatory pathways remain almost entirely undefined, and the solution for interactions governed by the intra- and extra-cellular environment in cellular biology could definitely offer more protocols for NPR improvement (Utsumi, 2008). Two plasmids containing genes of α-amylase and NPR was constructed respectively, and the B. subtilis strain carrying these plasmids at the same time produced predominantly the α-amylase and NPR with few contaminating extracellular enzymes (Reid et al., 1986). It was confirmed that a B. subtilis strain carrying a foreign protease gene as well as another extracellular protein gene, cloned from the same donor strain, would produce predominantly these two extracellular proteins. It may be due to gene dosage effect or pathway feedback regulation, but no further study was done. Since efficient transformation systems of Bacillus and other host are available, and more regulation genes for proteases production have been found, the information integration and the use of omic technology are beneficial for meticulous NPR improvement (Casanueva et al., 2010).

4.7. Stable Expression

Since plasmid expression vector is widely used in NPRs study and expression, a stable, high copy number hybrid plasmid carrying the protease gene is required. Most convenient vector plasmids have been derived from natural plasmids detected in Staphylococcus aureus such as pUB110, pC194,
and pE194 (Nguyen et al., 2005). Though these vector plasmids replicate stably in \textit{B. subtilis}, addition of recombinant DNA may cause structural and even segregational instability. Plasmids from \textit{Staphylococcus aureus} replicate as rolling circles, producing single-stranded DNA as an intermediate, and direct repeats within may lead to the deletion of one of the two repeats and the intervening DNA (Bron et al., 1991). This observation led to the utilization of homologous recombination. According to the molecular basis of their replication mode, the problem has been completely overcome by introducing plasmids using the theta-mode of replication, such as those derived from the natural plasmids pAMβ1, pTB19, pTA1060, and pBS72 (Nguyen et al., 2005; Phan et al., 2006). These plasmids provide great conviction for expression vectors construction afterwards (Nguyen et al., 2007; Zhang and Zhang, 2011).

5. Gene Mutations: Rational Engineering and Directed Evolution

During the past 20 years there has been a continuous flow of reports describing proteins improved on activity, specificity and especially stability by the introduction of mutations. These reports span a period from pioneering rational design work on small enzymes to protein design, and directed evolution. Compared to the engineering of subtilisin, a model for understanding the enormous rate enhancements affected by enzymes, date in existence is far from enough to understand the redesign of NPRs comprehensively. The stability (mainly measured as thermodynamic stability) has been the property of NPR which has been most amenable to enhancement, followed by properties of activity and specificity. Thermolysin, the prototype of neutral metalloproteases, is constantly used for comparisons. Regarding to the huge blank of our knowledge for enzyme improvement, directed evolution is still an effective solution for NPR improvement.

5.1. Thermodynamic Stability

At elevated temperatures, under non-inhibitory conditions, mature protein was rapidly degraded. Thermal inactivation of NPR (named thermolysin-like protease in some cases) follows first-order kinetics and is governed by a rate-limiting unfolding step that precedes the irreversible process of (rather complete) autolysis (Eijsink et al., 1992; Kidokoro et al., 1995; Vriend et al., 1998). Natural NPRs exhibit large differences in thermal stability (Veltman et al., 1997), making the interest on the study and engineering of protein stability lasting for a long time and yielding many variants (Eijsink et al., 2001). NPR has been subjected to extensive mutagenesis studies with the following major results:

(1) The mutational effects of various types of designed mutations in the C-terminal domain (which coincidently had been selected for these studies) were almost in the +1 to -1 \(^{\circ}C\) range (Eijsink et al., 1995).

(2) Systematic replacement of residues in NPR by the corresponding residue in thermolysin
yielded several mutants displaying relatively large stability effects (varying from -6.3 to +7.0 °C) (van den Burg et al., 1991; Veltman et al., 1996). Mutations with large effects were clustered in a surface-located region in the N-terminal domain of the protein and it is called a “weak spot” or “unfolding nucleus” (Eijsink et al., 2004). Single point mutations in weak spot could have dramatic effects on stability. Hence the discovery of a weak spot could contribute on successful stability improvement.

(3) It was suggested that unfolding process in the N-terminal domain plays an important role in thermal inactivation. An extremely stable variant with a half-life of 170 min at 100 °C was obtained by applying eight mutations on the thermolysin-like protease from B. stearothermophilus (Van den Burg et al., 1999), which is one of the most stable enzymes ever obtained by protein engineering. Another extreme stabilization of NPR was obtained by an engineered disulfide bond (Mansfeld et al., 1997). It is indicated that some of the mutations are of “entropic stabilization” or rigidification type, which affects the protein surface and surface interaction (Gromiha et al., 2002). These data are in agreement with breakdown products analysis, showing a preference for the generation of fissions at the N-terminal side of large hydrophobic residues (Van den Burg et al., 1990).

(4) Calcium binding is one of several mechanisms to create local stability. Most of the NPRs bind four calcium ions, they're related to autodegradation sites (Matsumiya et al., 2004). It has been shown that the calcium-dependency of stability is located in the unfolding region, and the destabilizing effect by mutations deteriorating this calcium site can be compensated by stabilizing mutations near the binding site (Veltman et al., 1998).

In summary, almost all mutations that improve stability could be contributed from surface-located residues, and many of the mutations that affect stability relate to weak calcium-binding sites, which are corresponding to the reports on subtilisin (Bryan, 2000).

5.2. Activity

Except for improved thermal stability, mutations which confer the enzymes desirable properties, such as enhanced catalytic activity, enhanced tolerance against organic solvent, altered substrate specificity, or altered pH-dependence of activity, have been identified (Kusano et al., 2010). With the evaluation of solvent composition on the NPR activity, which show that the activity depends on the dielectric constant of the reaction medium (i.e. neutral salts), it is suggesting the importance of surface charges of the molecule (Kusano et al., 2006). The ability to re-engineer enzymatic pH-activity profiles is of importance for industrial applications of enzymes, and enzymatic pH-activity profiles can be re-engineered by changing active site pKa values using point mutations. A few algorithm and software have been developed for biocatalysts engineering (Damborsky and Brezovsky, 2009). Site-directed mutagenesis of NPR has clarified the involvement of more and more amino acid substitutions and their combination in the catalysis such as Glu143 and His 231 in the active site and Gln 225 on the
molecular surface (Yasukawa and Inouye, 2007). However, tailoring the activity profile of NPR has not been achieved so far by site-directed mutagenesis.

5.3. Irrational Improvement: Classical Mutagenesis & Directed Evolution

Strain improvement by mutations is an age-old and successful method and it is still commonly used in protease alteration in the last decade, such as the application of the N\textsuperscript{+} ion implantation mutagenesis on A. oryzae for abundant protease production recently (Zhao et al., 2012). Chemical and physical mutageneses are usually simple to be carried out, conferring the classical mutagenesis competitiveness to some extent. Further, we’re illuminated to combine the mutations of different respects to obtain a variant enzyme with more desirable properties. However, negative correlation between the activity and the stability was observed, the same to a compromise in various enzymes, indicating mutations which increase enzyme activity accompanied by a decrease in protein stability and vice versa (Lee et al., 2006). In addition, it is not easy presently to predict the effect of mutational combination on enzyme properties (Mildvan, 2004).

Hence, the directed evolution (DE) of enzymes emerged recently is a key technology to generate enzymes with new and improved properties for their industrial applications. It’s a powerful strategy for altering enzyme by introducing substitutions with three approaches: DNA shuffling, random priming recombination and the staggered extension process (StEP) (Gupta et al., 2002). The DE starts with identification of a target enzyme and cloning the corresponding gene. With the assistance of an efficient expression system, the target gene is subject to random mutagenesis and recombination, thereby creating molecular diversity. This is followed by ultra-high throughput of screening and identification. Classical mutagenesis and DE could be called combinatorial approaches compared to rational design (Nixon and Firestine, 2000) because both of them consist of the creation of a library of random mutants of a particular protein, and no type of structural information or structure–function relationships are required. Actually, they offer a method for identifying such information. At present, the abundant researches on the subtilisin improvement with DE provide a successful instance for NPR improvement (Gupta et al., 2002).

6. Fermentation Optimization

6.1. Medium Formulation

Media which are generally used for protease production are rich in nitrogen sources, such as soybean meal, casein, gelatin, corn steep liquor, distiller’s solubles, brewer’s yeast, and carbohydrate sources such as starch, ground barley, or lactose (Blieva et al., 2003). 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 (Queiroga et al., 2012). Wheat bran and soybean meal are usually reported to be the best carbon and nitrogen source for NPR production. High carbohydrate concentrations repress enzyme production. Free amino acids also have a similar effect on protease production (Ivanitsa et al., 1978). Isoleucine and proline repress protease production by Bacillus sp. Peptides and proteins have the reverse effect, inducing protease production in a number of microorganisms (Sumantha et al., 2006). Each organism or strain has its own idiosyncratic, physicochemical and nutritional requirements for maximum enzyme production. And various researchers have reported that culture conditions promoting protease production are significantly different from the culture conditions promoting cell growth. Hence, optimization of medium composition has to be carried out to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation.

An important factor to be monitored while developing a production medium is the cost-effectiveness of the medium. This can be achieved by using cheaply available agro-industrial residues such as wheat bran, rice bran, sunflower meal, etc. (Haq and Mukhtar, 2004; Sandhya et al., 2005). Additives such as vitamins (e.g. biotin), growth promoters (e.g. 1-naphthyl acetic acid) enhance protease production (Tunga et al., 2001). Microorganisms generally prefer easily metabolizable carbon sources over alternative. Carbon repression, the repression of enzymes synthesis related to alternative carbon sources catabolism, is one of the mechanisms. It has been proven to be regulated by protein CREA (a negative-acting protein coded by the creA gene) (Ruijter and Visser, 1997) and various pathways (Ishida et al., 2000) in Aspergillus. It is beyond the scope of this review to explore in detail the potential of molecular mechanism on carbon metabolism to NPR production, thus an emphasis was placed on optimization of bioprocesses engineering.

6.2. Fermentation Technologies

NPRs have been produced in submerged fermentation (SmF) and solid-state fermentation (SSF) (Sandhya et al., 2005; Singhania et al., 2009). Both of them influence various aspects of the growth of the microorganism as well as enzyme production.

SmFs are usually carried out with a dissolved or suspended substrate in an aqueous medium. Different substrates affect the NPR activity significantly. While simple substrates such as Luria-Bertani medium (LB medium) yield low enzyme units, more complex substrates such as soybean meal and wheat bran result in higher protease activities. Supplementation of a nitrogen-rich medium with glucose also enhances protease production (Boer and Peralta, 2000). Many types of SmFs have been described, of which continuous cultures have proven to yield better enzyme units than batch fermentations (Vierheller et al., 1995), though spontaneous mutations to less productive strains occurs in the long term.
SSF gained a fresh attention from researches and industries since recent few years. It has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water. SSF appears to possess a few advantages such as higher fermentation productivity and end-concentration of product. To some extent it resembles the natural habitat of microorganism and is preferred choice for microorganisms to grow and secret protein, while SmF can be considered as a violation to their natural habitat. Proteases from *Aspergillus* sp. are yielded higher in SSF using wheat bran as the solid media with 43.6% moisture content than in SmF (Sandhya *et al.*, 2005). Fungi grown under SSF produce more spores and fruiting bodies, thus contributing to a distinction in the physiology, which has a positive influence on enzyme production (Ward *et al.*, 1985).

With regard to cost economics, SSF has been proved to be more efficient than SmF because of the high solid concentration. Lots of agro and agro-industrial residues have been exploited to analyse their potential to be used as substrates for protease production (Singhania *et al.*, 2008). Similar to SmF, a combination of different substrates may give higher enzyme yields than exclusive use. Fermentation of NPR produced by *A. oryzae* NRRL 2217 was optimized by the combined substrate, coconut oil cake and wheat bran in the mass ratio of 1:3 (Sumantha *et al.*, 2005). By the side of fermentation methods, drum, pot and tray method have proved very successful for *Aspergillus* fermentations (Singhania *et al.*, 2009). However, control mechanisms of gene expression in SSF have been poorly studied. On the comparison of the transcriptional regulation between two protease encoding genes in SmF and SSF of *A. oryzae* using wheat as a substrate, it was observed that in SSF the nutrients were directly consumed by the fungus after liberation from the substrate, while in SmF they were released in excess from the medium during fungal growth, and hence nutrient repression occurred, which in turn affected the expression of the enzyme (Te Biesebeke *et al.*, 2005).

Various modifications of SmF and SSF have been reported on protease production (Imanaka *et al.*, 2010). A membrane-surface liquid culture (MSLC) method was developed for *A. oryzae* fermentation (Feng *et al.*, 2006). In this method, a micro-porous membrane was provided for growth, one side of which was exposed to the air with the other side in contact with the liquid medium. Few reports have mentioned that proteases and amylases are concomitant enzymes in *Aspergillus*, and the yield of extracellular enzymes is significantly influenced by physicochemical conditions (Negi and Banerjee, 2006). For this reason, it may be advantageous and cost-effective to consider amylase and other enzymes as factors in fermentation. NPR and amylase could be used together in food industry, pharmaceuticals and detergent industry, etc. Extracellular protease production is greatly influenced by physical factors such as pH, temperature and incubation time and by others factors such as media composition and presence of metal ions (Thys *et al.*, 2006). Controlling the concentration of dissolved oxygen is a standard feature in fermentation processes. However, the measurement of dissolved CO₂ concentrations is often neglected. In advanced research room air supplemented with CO₂ resulted in a 43% raise in NPR activity by *B. caldolyticus* when compared with unsupplemented room air (Bader *et al.*).
6.3. Statistical Optimization

In the traditional biological system, optimization is done by “trial and error” or by changing one control variable at a time. This method is inefficient in finding the true optimum conditions because it has the limitations of ignoring the importance of interaction of various physiochemical parameters. Statistical optimization of fermentation parameters has been widely used for proteases production by *Bacillus* and *Aspergillus* (Deng et al., 2010; Gurunathan and Sahadevan, 2011). Factorial design, whose experimental units are taken on all possible combinations, is suitable for multi-factor and multi-level experiments. Hence, it provides an easy way for optimization of fermentation conditions. Modificatory methods have been used in specific cases. Evolutionary operation (EVOP) factorial design technique has been explored for NPR production in a single bioreactor by modified solid-state fermentation (Negi and Banerjee, 2006). It could be considered as a multivariable sequential search technique.

Appearance of relatively newer experimental designs such as radial basis function (RBF), artificial neural network (ANN), genetic algorithms (GA), and response surface methodology (RSM) have been used to study the relationship between various interacting parameters during the course of fermentation for enzyme production (Dutta et al., 2004). In a case study, ANN was indicated superior to RSM on sensitivity analysis and optimization abilities in capturing the non-linear behavior of the system (Desai et al., 2008). Statistical optimization has played a more important role in the application of NPR, like the optimization of hydrolysis conditions (Bhaskar and Mahendrakar, 2008).

7. Downstream Processing

A wide range of techniques are available for the recovery of the product from the fermented substrate depending on the source (liquid or solid form), scale of operation, enzyme stability, etc. The objectives of a design for the purification are usually including high degree of purity, high overall recovery of activity of enzyme, and reproducibility. Since NPRs are extremely sensitive towards autodigestion, conditions were chosen focusing on stabilization (Van Den Burg et al., 1989).

As an extracellular enzyme, for SSF, the fermented solids are crumbled and extraction in either a batch or continuous process. This is followed by enzyme leaching with water, aqueous buffers, diluted solutions of salts (e.g. 0.9% sodium chloride), glycerol (1%), or diluted (0.1%) solutions of non-ionic detergents such as Triton X-100 and Tween 20, etc. In liquid preparations, the enzyme is stabilized against chemical and microbial denaturation and degradation by the addition of high concentrations of salts such as ammonium sulphate and preservatives such as glycerol to increase product shelf life. The pH of the liquid should be adjusted.
There’re activating effect of divalent cations (Mg$^{2+}$, Ca$^{2+}$, Fe$^{2+}$) and inhibiting effect of certain chelating agents (EGTA, EDTA) on metalloprotease (Sumantha et al., 2005). Inhibitory effect of Co$^{2+}$ has also been reported. Specific NPRs might also be stabilized by the addition of divalent cations, which act as either oxidizing or reducing agents (Sumantha et al., 2006). While some of these additives may also act as preservatives, specific anti-microbial preservatives are also added. NPRs that show greater stability in solid form like thermolysin are spray-dried to obtain the powdered form, while others are used as liquid. In addition, the effects of solvent composition on the activity and reported remarkable activation and stabilization have been evaluated by high concentrations of neutral salts, crystallographic analysis in the presence of 4 M NaCl, the activation by cobalt-substitution of the catalytic zinc ion, and inhibitory effects of alcohols (Kusano et al., 2006).

Purification of proteases to homogeneity is a prerequisite for studying their mechanism of action. Vast numbers of purification procedures for proteases, involving affinity chromatography, ion-exchange chromatography, and gel filtration techniques, have been well documented. Cooperative enzymes system is promising, considering that purification of different enzymes is generally tedious and costly when they are purified individually from different fermentation systems, and their combined activities maybe greater than the sum of their individual activities. There’re few reports on cooperative enzymes comprising alkaline or mixtures of alkaline and NPRs without stabilizers (Stanislawski and Wiersema, 1985). As described above, producing amylase and NPR in a single reactor might be a case of inspiration.

8. Final Considerations

NPRs have found a wide range of applications in various industries. Because of the sensitivity of mesophilic metalloprotease to temperature and chelant, it’s advantageous for controllable hydrolysis degree. The thermal stable protease and thermolysin are potential to combine with alkaline proteases or acid proteases to provide broad range of pH activity or thermal stability. Even though the production has been improved significantly by the utilization of hyper-producing strains and empirically proven fermentation technologies, efforts are still being done to find newer sources of enzymes, better production techniques and novel applications of these enzymes in unexplored fields. Along the way, many strategies have been proposed, and some of them have reasonable success rates, including gene mutations on property adjustment (e.g. ultra-thermal stability), discovering regulation genes in transcriptome and secretome, utilization of agro- or agro-industrial residual.

At the base that strain improvement by genetic engineering has been established as a good alternative, plasmid expression vector constituted of strong promoter as well as signal peptide of high secreting efficiency, along with hosts in turning up corresponding genes in regulatory network or deficiency of irrespective extracellular proteins, is a promising strategy for strain construction.
Regarding to the gene itself, screening for novel enzymes provides fundamental resources on mechanism studies of rational engineering and directed evolution, whose successful usage on various enzymes are good model system such as subtilisin engineering. Continuation of these emerging technologies may provide additional information that will be of importance for the improvement of NPR. Down to fermentation and purification, since they’re comparative developed and highly dependent on producing strains, improvement on cost-efficiency and eliminating of physicochemical repression factors should be encouraged.

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