

Enzymatic lysis of microbial cells

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Abstract Cell wall lytic enzymes are valuable tools for the biotechnologist, with many applications in medicine, the food industry, and agriculture, and for recovering of intracellular products from yeast or bacteria. The diversity of potential applications has conducted to the development of lytic enzyme systems with specific characteristics, suitable for satisfying the requirements of each particular application. Since the first time the lytic enzyme of excellence, lysozyme, was discovered, many investigations have contributed to the understanding of the action mechanisms and other basic aspects of these interesting enzymes. Today, recombinant production and protein engineering have improved and expanded the area of potential applications. In this review, some of the recent advances in specific enzyme systems for bacteria and yeast cells rupture and other applications are examined. Emphasis is focused in biotechnological aspects of these enzymes.

Keywords Bacteriolytic · Cell lysis · Intracellular protein recovering · Yeast-lysing enzyme

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Bacteriolytic enzymes

Bacteriolytic enzymes have been greatly used in the biotechnology industry to break cells. Major applications of these enzymes are related to the extraction of nucleic acids from susceptible bacteria and spheroplasting for cell transformation (Table 1). Other applications are based on the antimicrobial properties of bacteriolytic enzymes. For instance, creation of transgenic cattle expressing lysostaphin in the milk generated animals resistant to mastitis caused by streptococcal pathogens and *Staphylococcus aureus* (Donovan et al. 2005). Since this peptidoglycan hydrolase also kills multiple human pathogens, it may prove useful as a highly selective, multipathogen-targeting antimicrobial agent that could potentially reduce the use of broad-range antibiotics in fighting clinical infections.

The use of lytic enzymes for the release of recombinant proteins from bacteria has been successful in many cases. For instance, Yang et al. (2000) used a temperature-sensitive lytic system for efficient recovery of recombinant proteins from *Escherichia coli*, and Zukaite and Bizilevicius (2000) applied lytic enzymes to accelerate the production of hyaluronidase by recombinant *Clostridium perfringens* in the course of batch cultivation.

Table 1 Present and potential applications of microbial lytic enzymes

Application	References
<i>Bacteriolytic enzymes</i>	
DNA extraction from Gram-positive bacteria	Niwa et al. (2005), Ezaki et al. (1990)
Production of transgenic cattle resistant to microbial infections	Kerr and Wellnitz (2003), Donovan et al. (2005)
Antimicrobial for medical and food applications	Masschalck and Michiels (2003), Loeffler et al. (2001), Fischetti (2003), Sava (1996)
Release of recombinant proteins	Zhang et al. (1999), Zukaite and Bizilevicius, (2000), de Ruyter et al. (1997)
<i>Yeast-lysing enzymes</i>	
Preparation of protoplasts, cell fusion, and transformation of yeast	Kitamura (1982)
Production of intracellular enzymes	Zomer et al. (1987)
Pre-treatment to increase yeast digestibility	Kobayashi et al. (1982)
Preparation of soluble glucan polysaccharides	Jamas et al. (1986)
Alkali extraction of yeast proteins	Kobayashi et al. (1982)
Production of yeast extracts	Conway et al. (2001)
Food preservation	Scott et al. (1987)
Release of recombinant proteins from <i>Saccharomyces cerevisiae</i>	Asenjo et al. (1993) Ferrer et al. (1996)

Bacterial cell wall

Based on the cell wall structure, bacteria are divided in Gram-positive and Gram-negative. Chemical composition and structure of the peptidoglycan in both types of bacteria are similar, though are much thinner in the Gram-negatives. The peptidoglycan layer, a polymer of *N*-acetyl-D-glucosamine units $\beta(1 \rightarrow 4)$ -linked to *N*-acetyl-muramic acid, is responsible for the strength of the wall (Koch 1998). In Gram-positive bacteria, multiple layers of peptidoglycan are associated by a small group of amino acids and amino acid derivatives, forming the glycan-tetrapeptide, which is repeated many times through the wall. Penta-glycine bridges connect tetrapeptides of adjacent polymers. Towards the out side, peptidoglycan is connected to teichoic acids and polysaccharides. Gram-negative bacteria have a two-layer wall structure with a periplasmic space between them: an outer membrane composed of proteins, phospholipids, lipoproteins and lipopolysaccharides covers the inner, rigid peptidoglycan layer.

In Gram-negative bacteria, the outer membrane precludes the access to lytic enzymes, causing the major difference regarding to lytic procedures. Lysozyme by itself can lyse Gram-

positive bacteria, but pre-treatment with a detergent (e.g. Triton X-100) or a cation chelating agent (as EDTA) is usually necessary to remove the outer membrane of Gram-negative cells.

Lysozyme, autolysins and endolysins

Enzymes that digest peptidoglycan of bacteria are collectively called murein hydrolases. Based on their bond specificity, they are classified as: (i) glycosidases, which split polysaccharide chains (lysozymes or muramidases and glucosaminidases), (ii) endopeptidases, which split polypeptide chains, and (iii) amidases, which cleave the junction between polysaccharides and peptides. Among muramidases, lysozyme is the best known and best described; it is produced mostly from hen egg white. Gram-negative bacteria, including some major foodborne pathogens, are usually insensitive to lysozyme. In consequence, besides the use of Triton X-100 or EDTA, several other strategies have been developed to expand the uses of lysozyme to Gram-negative bacteria. These include: denaturation of lysozyme (Touch et al. 2003); modification of lysozyme by covalent attachment of polysaccharides (Aminlari et al. 2005) or fatty acids (Ibrahim et al. 1991); attachment of hydrophobic peptides to the C-terminal

(Ibrahim et al. 1994) or cell permeabilization by high hydrostatic pressure (Masschalck et al. 2001).

Extensive hydrolysis of peptidoglycan by lysozyme results in cell lysis and death in a hypo osmotic environment. Some lysozymes can kill bacteria by stimulating autolysin activity upon interaction with the cell surface (Iacono et al. 1985). In addition, nonlytic bactericidal mechanism, involving membrane damage without hydrolysis of peptidoglycan, has been reported for c-type lysozymes, including human lysozyme (Laible and Germaine 1985) and hen egg white lysozyme (HEWL) (Ibrahim et al. 2001; Masschalck et al. 2002). An increasing body of evidence supports the existence of a nonenzymic and/or nonlytic mode of action of lysozyme (reviewed in Masschalck and Michiels 2003).

Even if lysozymes from many different sources have been isolated and characterized, HEWL is the only lysozyme that is currently used in most of the commercial applications. HEWL is produced abundantly from their natural source, is inexpensive and has a wider working range than several other lysozymes.

Efforts have been carried out for recombinant expression of lysozyme in *E. coli*; however, in this host HEWL is produced inactive as inclusion bodies (Schlörb et al. 2005). More successful have been the attempts of expression in different strains of *Aspergillus niger* (Archer et al. 1990; Mainwaring et al. 1999; Gyamerah et al. 2002; Gheshlaghi et al. 2005).

HEWL is the most used lysozyme for bacterial cell wall disruption. However, some Gram-positive bacteria are resistant to lysozyme. In those cases, autolysins and endolysins can be applied. Autolysins digest the cell wall peptidoglycan from cells that produce them and of some other related bacteria; they are mainly produced by Gram-positive bacteria and participate in a growing number of biological processes (Smith et al. 2000). Originally described some years ago, only in the past 10 years the molecular basis of their functions and mechanisms have developed enough to allow the exploitation of these enzymes potential for medical and biotechnological applications (Table 1). Gram-positive, lysozyme-resistant bacteria are usually lysed by cognate

autolysins and by autolysins from related bacteria.

Autolysins are ubiquitous enzymes but the best characterized are those from *Bacillus subtilis* (Smith et al. 2000), *Staphylococcus aureus* (Foster 1995), and *Streptococcus pneumoniae* (Lopez et al. 2000). Typically, autolysins have a modular structure, with a *N*-terminal signal peptide followed by a second domain, which contains the active site. In addition, these proteins harbor repeat motifs flanking either the *N*- or *C*-terminal of the catalytic domain.

Endolysins (or lysins) are lytic enzymes that are functionally related to autolysins except they are phage-encoded enzymes. They digest bacterial peptidoglycan at the terminal stage of the phage reproduction cycle, allowing the release of the viral progeny out of the cell. Recent research has not only revealed the diversity of these hydrolases, but also yielded insights into their modular organization and their three-dimensional structures (Fischetti 2005). Lysins can either be endo β -*N*-acetylglucosaminidase or *N*-acetylmuramidase, endopeptidase or amidase. Most of the endolysins are not translocated through the cytoplasmic membrane to attack their substrate in the peptidoglycan; this action is controlled by holin, enzyme produced by the same phage lytic system, that disrupts the membrane and permits the access of the lytic enzyme to the peptidoglycan (Wang et al. 2000).

Owing to their specificity and high activity, endolysins have been employed for various in vitro and in vivo aims, such as food science, microbial diagnostic and for treatment of experimental infections (Loessner 2005; Borysowski et al. 2006). They are potentially useful antimicrobial agents for elimination of the opportunistic pathogen *Listeria monocytogenes* from foods and food-grade ingredients (Loessner 2005).

Lysin LysK, from the anti-staphylococcal phage K, and bacteriophage-lytic lysin from *Listeria monocytogenes* were cloned and expressed in the Gram-positive *Lactococcus lactis*, resulting in the production of active and functional lysin (O'Flaherty et al. 2005; Gaeng et al. 2000). Lysin from an anti-listeria bacteriophage was also highly expressed as an active enzyme in *E. coli* (Loessner et al. 1996).

The activity spectrum of lysins is mainly restricted to the host bacterial species of the phage from which the endolysin was derived, and also depends on the unique linkages to be cleaved in the cell wall, the specific enzyme activation by components present exclusively in the cell wall, and on the specificity in substrate recognition and cell wall binding.

Other bacteriolytic enzymes

Many other lytic enzymes are able of lysing lysozyme-resistant Gram-positive bacteria. There are: achromopeptidase from *Achromobacter lyticus* M497-1, a broad spectrum bacteriolytic enzyme lysing *Staphylococcus aureus* and *Micrococcus luteus* (Li et al. 1998); lysyl-endopeptidase produced by *Lysobacter* sp. IB-9374 (Chohnan et al. 2002; Ahmed et al. 2003); labiase, secreted by *Streptomyces fulvissimus*, used for bacterial DNA/RNA extraction from *Lactobacillus*, *Aerococcus* and *Streptococcus* (Niwa et al. 2005). Labiase and achromopeptidase purified from their respective native sources are commercially available from Sigma-Aldrich and Wako Pure Chemical Industries; mutanolysin, a *N*-acetyl muramidase like lysozyme, derived from *Streptomyces globisporus*, can hydrolyze peptidoglycan from *Listeria*, *Lactococcus*, *Lactobacillus*, *Pneumococcus* and other streptococci; and lysostaphin, which specifically cleaves the cross-linking pentaglycine bridges in the cell wall of staphylococci strains (Recsei et al.1987).

Yeast-lysing enzyme systems

Yeast cell wall

The yeast cell wall is a highly dynamic structure, responsible for protecting the cell from rapid changes in external osmotic potential. Several comprehensive reviews on this subject have been published (Kollar et al. 1997; Lipke and Ovalle 1998). The yeast wall is composed mostly of mannoprotein and fibrous $\beta(1 \rightarrow 3)$ glucans with some branches of $\beta(1 \rightarrow 6)$ glucans. The glucans are essential components, responsible for the mechanical strength, shape and elasticity of the

cell wall. The $\beta(1 \rightarrow 3)$ glucan–chitin complex is the major constituent of the inner wall and forms the fibrous scaffold of the wall. On the outer surface of the wall are the mannoproteins, which are densely packed and limit wall permeability to solutes. $\beta(1 \rightarrow 6)$ glucan links the components of the inner and outer walls. All these components are covalently linked to form macromolecular complexes, which are assembled in unit modules, each built around a molecule of $\beta(1 \rightarrow 3)$ glucan. A minority of modules has chitin (a $\beta(1 \rightarrow 4)$ linked polymer of *N*-acetylglucosamine) chains attached to $\beta(1 \rightarrow 3)$ or $\beta(1 \rightarrow 6)$ glucan. The modules are associated by noncovalent interactions in the glucan–chitin layer and by covalent cross-links in the mannoprotein layer, including disulfide bonds between mannoproteins.

Yeast-lysing enzyme systems: action mechanism

Enzyme systems for yeast cell lysis are usually a mixture of several different enzymes, including one or more $\beta(1 \rightarrow 3)$ glucanase (lytic and non-lytic), protease, $\beta(1 \rightarrow 6)$ glucanase, mannanase and chitinase, which act synergistically for lysing the cell wall.

Enzymatic cell lysis of yeast begins with binding of the lytic protease to the outer mannoprotein layer of the wall. The protease opens up the protein structure, releasing wall proteins and mannans and exposing the glucan surface below. The glucanase then attacks the inner wall and solubilizes the glucan. In vitro, this enzyme cannot lyse yeast in absence of reducing agents, such as dithiothreitol or β -mercaptoethanol, because the breakage of disulphide bridges between mannose residues and wall proteins is necessary for appropriate exposition of the inner glucan layer. When the combined action of the protease and glucanase has opened a sufficiently large hole in the cell wall, the plasma membrane and its content are extruded as a protoplast. In osmotic support buffers containing 0.55–1.2 M sucrose or mannitol, the protoplast remains intact, but in dilute buffers it lyses immediately, releasing cytoplasmic proteins and organelles, which may themselves lyse. Meanwhile, proteins released from the wall and the cytoplasm could be subject

to attack by product-degrading protease contaminants in the lytic system or in the yeast cells themselves.

Source of yeast-lysing enzymes

A number of different microorganisms produce extracellular yeast-lysing enzymes. These organisms exhibit predatory activity against yeast and other microbial cells and have been isolated from diverse habitats. From a commercial viewpoint, the most important are *Cellulosimicrobium cellulans* (former *Oerskovia xanthineolytica*, also known as *Arthrobacter luteus*), *Cytophaga* sp. and *Rhizoctonia* sp. A lytic system from *Rhizoctonia solani* is also commercially available (Kitalase), containing $\beta(1 \rightarrow 3)$ endoglucanase, protease, pectinase and amylase activities. Other prokaryotes recently discovered as producers of lytic enzymes are *Micromonospora chalcea* (Gacto et al. 2000) and *Lysobacter enzymogenes* (Palumbo et al. 2003). Some biochemical properties of the most important yeast-lysing enzymes are given in Table 2.

Yeast-lysing enzymes

Lytic β -1,3-endoglucanases

Frequently lytic complexes have multiple β -glucanases, with different substrate specificity and mode-of-action patterns. Lytic $\beta(1 \rightarrow 3)$ glucanases catalyze the hydrolysis of the $\beta(1 \rightarrow 3)$ link present in laminarin, pachyman, curdlan and yeast wall glucan. Molecular aspects of lytic endoglucanases from the major source, the actinomycete *Cellulosimicrobium cellulans*, have been recently reviewed (Ferrer 2006). Practically, there exist three $\beta(1 \rightarrow 3)$ glucanases from two different strains of *Cellulosimicrobium cellulans*; strain ATCC 21606, former strain 73/14 (Scott and Schekman 1980; Shen et al. 1991) produces a 64 kDa glucanase with high lytic activity on yeast cell wall (Table 2). The enzyme is commercially known as lyticase or zymolyase and is available as a mixture with protease, which improves the yeast lysis results. A recombinant version is also available (Shen et al. 1991).

Table 2 Yeast-lysing enzymes

Source	Optimum pH	Optimum temperature (°C)	Molecular weight ($\times 10^{-3}$)	References
<i>Cellulosimicrobium cellulans</i> ATCC 21606 ^a				
Glucanase	8.0		55	Scott and Schekman (1980), Shen et al. (1991)
Protease	>10.0			
<i>Cellulosimicrobium cellulans</i> DSM 10297 ^b				
Glucanase	8.0	40 ^c	41	Salazar et al. (2001), Ventom and Asenjo (1990)
Protease	9.5–10.0		11–23	
<i>Cellulosimicrobium cellulans</i> TK-1				
Glucanase	7.5		40	Saeki et al. (1994)
<i>Rhizoctonia</i> sp.				
Glucanase	6–7	40 ^d		Kobayashi et al. (1981)
<i>Arthrobacter</i> sp.				
Protease	11	55 ^e		Adamitsch et al. (2003)
<i>Rarobacter faecitabidus</i>				
Protease I			34	Shimoi et al. (1992)

Symbols

^a Also known as *Arthrobacter luteus* 73/14

^b Also known as *Oerskovia xanthineolytica* LL G109

^c Over 30 min, using laminarin as substrate

^d Using viable *Saccharomyces cerevisiae* cells as substrate

^e Over 30 min, using azocasein as substrate

The other important producer of lytic $\beta(1 \rightarrow 3)$ endoglucanase is *Cellulosimicrobium cellulans* DSM 10297 (former *Oerskovia xanthineolytica* LL-G109) (Table 2); this strain produces β -glucanase II (Bgl II) and Bgl II_A (a glucanase lacking the mannose binding domain) but only Bgl II has lytic activity (Ferrer et al. 1996; Salazar et al. 2006). Evaluation of the yeast lytic activity of Bgl II was originally accomplished using the enzyme isolated from the supernatant of a *Cellulosimicrobium cellulans* culture, where the enzyme is found mainly as a truncated form, lacking the C-terminal mannose-binding domain. The absence of the mannose-binding domain may be the explanation for the lower lytic activity observed in Bgl II, not really a consequence of differences in the functional properties of the catalytic domains.

Lytic proteases

Proteases act initially on the yeast surface, allowing the exposition of the glucan layer to the glucanases hydrolytic action (Ventom and Asenjo 1991; Adamitsch et al. 2003). Lytic proteases have a characteristic high affinity for the yeast wall surface and often have anomalously low activities against conventional protein substrates. The two proteases isolated from the *Cellulosimicrobium cellulans* extracellular lytic complex are similar in molecular weight (about 12 kDa) and optima pH for azocasein hydrolysis (9–10) (Table 2) (Ventom and Asenjo 1990).

Rarobacter faecitabidus produces a major lytic protease (Shimoi et al. 1992). This enzyme has a mannose-binding domain in the C-terminal region, homologous to the mannose-binding domain of the *Cellulosimicrobium cellulans* $\beta(1 \rightarrow 3)$ glucanase. This domain allows the attachment of the enzyme to the mannoproteins in the yeast cell surface, increasing the local enzyme concentration and facilitating the lysis. Despite of the important role of yeast-lysing proteases in nature, in vitro these enzymes can be effectively replaced by reducing compounds, such as β -mercaptoethanol (Ventom and Asenjo 1990), which destroy disulphide bridges that bind mannoproteins in the yeast surface.

Specificity of yeast-lysing enzymes

Specificity of lytic enzymes is determined by interactions produced between the carbohydrates in the cell wall and amino acids in the enzyme located either in the active site or in the carbohydrate-binding module. In general lytic systems are specific for different microorganisms. For example, the lytic systems of *Cellulosimicrobium cellulans* strains are specific for yeast, and lysozyme is specific for bacteria. One exception to this is the lytic system of *Cytophaga* NCIB 9497, which has a broad activity spectrum, having high activity on both yeast and Gram-positive bacteria (Le Corre et al. 1985).

Usually, lytic enzymes that are able to digest *Saccharomyces* sp. cell wall, such as zymolyase (Kitamura and Yamamoto 1972) and lyticase (Scott and Schekman 1980) from *Cellulosimicrobium cellulans*, can also lyse yeast from the genera of *Candida*, *Hansenula* and *Pichia*, although in some cases higher enzyme concentration is required for satisfactory yields. In general, susceptibility of filamentous fungi to yeast-lysing enzymes is smaller (Loffler et al. 1997), which is probably a consequence of differences between yeast and filamentous fungi cell wall structures. For fungal protoplast formation, commercial preparations of lytic enzymes from the fungus *Trichoderma harzanium* are most frequently preferred.

Production of yeast-lysing enzymes

Lytic $\beta(1 \rightarrow 3)$ glucanases from *C. cellulans* strains ATCC 21606 and DSM 10297 have been expressed in *Escherichia coli* (Shen et al. 1991; Salazar et al. 2001). Both recombinant enzymes were abundantly produced and released to the extracellular space. Kinetic properties of both recombinant enzymes were indistinguishable from those of the native enzymes. Recombinant glucanase from strain ATCC 21606 is commercially available from Krackeler Scientific Inc as Quantazyme ylg, which, it is stated, is the only protease-free glucanase commercially available. Recently, random mutagenesis of Bgl II produced a variant having three times more specific activity

in substrate laminarin and also in *Saccharomyces cerevisiae* cell wall, which is an interesting promise of further applications of Bgl II in protein extraction operations from yeast (Salazar et al. 2006).

Applications of yeast-lysing enzymes

Yeast-lysing glucanases have an enormous field of applications. Some of them are currently very well documented and developed, as the use for nucleic acid extraction from yeast and transformation; others are just beginning to be implemented. Most important applications of lytic glucanases are depicted in Table 1. The next paragraphs describe the use of yeast-lysing glucanases for modulating the cell wall permeability as a first step in a downstream process for protein recovering from yeast.

Many of the proteins produced by recombinant bacteria and yeast remain intracellular and are not secreted by the microbial cell. The use of lytic enzymes for cell disruption and product release was suggested some years ago to solve this problem and for avoiding some critical drawbacks that traditional mechanical methods have (i.e. unspecificity, proteolysis, contamination, high viscosity, amongst others). Cell lysis by enzymes is a convenient procedure since may conduct to permeabilization and differential product release. Such methods can achieve an important purification at an early stage, hence simplifying the initial downstream recovery steps considerably. Besides, the use of lytic enzymes offers the possibility of designing a gentle process for differential product release. By using purified, protease-free glucanase enzyme it has been possible to carry out controlled *Saccharomyces cerevisiae* lysis, which results in the selective release of cloned (recombinant) intracellular protein particles (Asenjo et al. 1993).

Specific activity of the lytic enzymes is a key factor that has to be considered when analyzing the possibility of using lytic enzymes for cell disruption and product release. It has to be sufficiently high to obtain fast cell breakage without allowing endogenous intracellular proteases degrading the product. Production of engineered, optimized, purified $\beta(1 \rightarrow 3)$ glucanases

seems to be essential for application in extraction procedures of recombinant proteins from yeast, without the necessity of including proteases to improve the process. In this sense, results of Salazar et al. (2006) are promising. By means of random mutagenesis and high-throughput screening they produced improved variants of the *Cellulosimicrobium cellulans* DSM 10297 lytic $\beta(1 \rightarrow 3)$ glucanase, whose specific lytic activity on viable *Saccharomyces cerevisiae* cells rose almost three times compared to the wild-type enzyme. All the responsible mutations are located in a small space of sequence, suggesting that the region could be a target for further improvements of the lytic activity. These results demonstrate that molecular evolution methods are suitable for optimization of the Bgl II hydrolytic activity.

The aforementioned application of $\beta(1 \rightarrow 3)$ glucanases for protein extraction from yeast considers the incubation of the cells with exogenously added lytic enzymes. A different approach has been the construction of yeast strains expressing $\beta(1 \rightarrow 3)$ glucanase under the control of a regulated promoter, hence modulating the cell wall porosity by controlled degradation of the wall (Zhang et al. 1999).

Summary and conclusions

In this article we have reviewed the enzymes presently available for cell lysis of bacteria and yeast. Lytic enzyme systems are usually specific either for yeast or for different types of bacteria.

Sources of lytic enzymes and latest developments on enzyme production have been reviewed. Specific applications developed in the last years for lytic enzymes have been examined. The design and use of lytic enzyme systems for differential product release from microbial cells have been reviewed.

These studies show that the use of lytic enzyme systems has tremendous promise as a method of controlled lysis and differential product release.

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