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Enzybiotics: New Weapon in the Army of Antimicrobials: A Review

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ABSTRACT

As promising antibacterials, endolysins own several pertinent features viz., diverse novel mode of action, antibacterial spectrum, low probability of developing resistance and being highly active with explicit specificity against host bacteria. Bacteriophage endolysins are mureolytic enzymes which facilitate direct targeting of peptidoglycan bonds in the bacterial cell wall. Encoded by the bacteriophage genome they are synthesized at the end of the phage lytic life cycle, headed for lysing host cell and releasing newly produced virions. In addition to this "lysis from within", endolysins from phages of gram-positive hosts are also able to swiftly lyse bacteria upon exogenous application. Lysozyme as well as endopeptidase like lysostaphine have been recommended in neonatal streptococcal and staphylococcal infection, respectively. Literature reveals strong potential of phage enzymes in human health care and veterinary medicine for control of pathogens and treatment of diverse systemic infections. They have wide applications in pathogen detection and development of diagnostics, as a means of biodefence, eliminating food pathogens and in control of phytopathogens. The defensins and cathelicidins can be exploited as enzybiotics among other families of antimicrobial peptide gene. In innate immunity such antibiotic peptides that are endogenous in nature play crucial role and forms first line of defense for protecting internal as well as external body surfaces of the host. The important portals of enzybiotics (EnzyBase and phiBIOTICS) are playing crucial role for disseminating the state of knowledge of enzybiotics. The present review discusses the widespread potential of various bacteriophage lysins/enzybiotics in the perspective of future antibacterial drug development.

Key words: Endolysins, bacteriophage, lysins, resistant bacteria, bacterial infections, antimicrobial, therapy, human, animals

INTRODUCTION

Day by day increasing incidence of antimicrobial resistance and lack of cent-percent effective treatment modalities has ignited a renewed search of novel antimicrobials. Emerging antibiotic resistance, climate changes/global warming, increasing immunocompromised patients and one

health concept (Okeke *et al*., 2005; Dhama *et al*., 2013a, b; Tiwari *et al*., 2013a) demands novel/alternative therapeutic regimens. Such treatment modalities include phages (bacteriophages, mycophages), avian egg antibodies, probiotics, panchgavya (cowpathy), cytokines, monoclonal antibodies, stem cells, herbs, ethno-veterinary medicines, nutritional immunomodulation and others (Breithaupt, 1999; De Groot and Scott, 2007; Hanlon, 2007; Kusuma *et al*., 2007; Mahima *et al*., 2012, 2013a, b; Amarpal *et al*., 2013; Dhama *et al*., 2008, 2011, 2013c, d, e; Tiwari *et al*., 2011, 2012, 2013b, 2014a, b). To overcome the hurdles of microbial/bacterial resistance these various alternatives/emerging therapeutics are gaining momentum which are developing new boulevard to brawl with resistant superbugs. In the series of therapeutic molecules and new emerging therapies recent approach is of exploiting bacteriophage enzymes, commonly known as endolysins and popularized as enzybiotics or endolysin therapy. Biochemically, bacteriophage endolysins (Ply) are peptidoglycan hydrolases enzymes encoded by double-stranded DNA bacteriophages, produced in phage-infected bacterial cells toward the end of their replicative lytic cycle in order to degrade the peptidoglycan of the host cell from inside. This leads to bacterial lysis and subsequent release of progeny phages. First report of successful *in vivo* application of endolysins regarding prevention of bacterial infection and its elimination was documented in 2001 (Schuch *et al*., 2002). Lysins have been found to be potential in reducing the chances of vaginal and oropharyngeal infections caused by *Streptococcus agalactiae* and even infection due to virulent organism like *Bacillus anthracis* (Cheng *et al*., 2005; Low *et al*., 2005).

Two proteins, an endolysin and a holin, are crucial for bacteriophage induced bacterial lysis. Holins create membrane pores so that endolysins reach and cleave the peptidoglycan, thus inducing lysis and death of the bacterial cell. Term enzybiotic signifies the role of phage enzymes as an antibiotic against bacterial infections (Lopez *et al*., 2004; Hermoso *et al*., 2007). As no outer membrane is present in the Gram-positive bacteria, peptidoglycan become more susceptible to the action of endolysins due to unrestricted access when applied externally (as purified recombinant proteins) and destroy such microorganisms rapidly. This renders endolysins as interesting antimicrobial candidates, particularly in current scenario of rising bacterial drug resistance (Low *et al*., 2011; Tiwari *et al*., 2013a, 2014a, b). Endolysins being specific peptidoglycan hydrolases, reduce the incidences of antibiotic-resistant pathogens rather than merely acting as broad-range antimicrobials. The feature of endolysins targeting unique and highly conserved bonds of peptidoglycan retards the probability of developing resistance against the activity of bacteriophage endolysins (Loeffler *et al*., 2001; Sandeep, 2006). The inimitable capability of endolysins to quickly cleave peptidoglycan in host species specific manner makes them promising potential antibacterial agents (Wang *et al*., 2000; Moak and Molineux, 2004; Fischetti *et al*., 2006; Fenton *et al*., 2010; Schmelcher *et al*., 2012). This review reveals information on structure of these enzymes, their mechanism of action and a special focus on their lytic activity and potential as antimicrobials against various bacterial agents particularly against Gram-negative and intracellular pathogens. Strategies for optimizing endolysins for their specific and most recent beneficial applications in the field of medical and veterinary sciences, health sectors, agriculture, in the arena of food safety and biotechnology by highlighting new developments on these antimicrobial proteins have also been discussed.

MECHANISM OF ACTION

Bacteriophages, depending upon structure follow two methods to release their progeny virions from host bacterial cells. Filamentous phage are released through bacterial cell walls without killing

bacterial cell. Non-filamentous phages make use of specific lysine enzymes to either inhibit the synthesis of peptidoglycan (single stranded RNA or DNA phage encoded enzymes) in the cell wall of bacteria or hydrolyze the built peptidoglycan by means of a holin-endolysin system (double stranded DNA phage encoded enzymes). Endolysins need a second protein holin to find their substrate molecule in the cell wall. Lysin remains in the cytosol till the late stage of the lytic cycle and hydrolyse the peptidoglycan of the bacterial cell wall when holinsform pores in the inner membrane of the infected host cell. This results in access of lysin to the peptidoglycan causing rapid cell lysis thus releasing mature phage progeny (Young, 1992; Wang *et al*., 2000).

In holin-endolysin system, phage requires both the holin and lysin for host cell lysis. Nevertheless, when lysins are employed as recombinant enzymes and applied exogenously to Gram-positive bacteria they are well capable of causing rapid lysis as no outer membrane is present to inhibit their access to the cell wall. In Gram-negative bacteria, the use of endolysin as antibacterial is limited as outer membrane hinders the access of exogenous lysins towards the cell wall peptidoglycan. Phage lysins selectively target specific pathogenic bacteria without affecting surrounding commensal microflora due to narrow host range (Loessner *et al*., 1995; Loessner, 2005).

Bacteriophage murein hydrolase enzymes display high specificity towards the cell wall of host bacteria due to presence of well defined cell wall binding domain that affix the endolysin to its substrate. Bacteriophage induces host lysis with the help of two proteins, endolysin and holin. Endolysin, a kind of muralytic enzyme accumulate in the cytosol during the vegetative cycle and degrade the bacterial cell wall with the help of holin proteins which are accrued inside the cytoplasmic membrane. Holins as membrane proteins remain in the membrane until a specific programmed time when the membrane becomes abruptly permeable to the endolysins. Destruction of the murein of cell wall and cellular bursting are immediate consequences of lytic action of endolysins. As holin genes direct the length of the infective cycle of lytic phages by means of holin proteins hence they are subject of deep evolutionary interest. Though action of holins is regulated by a number of diverse proteins, they represent one of the most sundry functional groups, with more than 100 known or putative holin sequences (Young and Blasi, 1995; Wang *et al*., 2000).

Lysis of the host cell with the Lyz endolysin of bacteriophage P1 is mediated by an N-terminal Transmembrane Domain (TMD), without involving a holin. The N-terminal domain of Lyz is capable of exporting the endolysin to the membrane but also facilitates its release into the periplasm. The unusual N-terminal domain functions as a signal-arrest-release sequence, which first acts as a normal signal-arrest domain to direct the endolysin to the periplasm in membrane-tethered form and then allows it to be released as a soluble active enzyme in the periplasm (Xu *et al*., 2004).

PlyPSA is another (314 amino acid) endolysin obtained from the temperate *Listeria monocytogenes* phage PSA with two polypeptide domains responsible for cell wall binding and enzymatic activities. PSA endolysin specifically recognize *L. monocytogenes* cells. The N-acetylmuramoyl-l-alanine amidase moiety is core formed by a twisted, six-stranded **β**-sheet flanked by six helices, while catalytic domain is highly similar to known phosphorylase/hydrolase-like a/**β**-proteins, including an autolysin amidase from *Paenibacillus polymyxa*. On the contrary, the C-terminal domain of PlyPSA attributes a novel fold, comprising two copies of a **β**-barrel-like motif, which are held together by means of swapped **β**-strands. The architecture of the enzyme with its two separate domains elucidates its exclusive substrate recognition properties and elaborates the lytic mechanisms of Listeria phage endolysins, special enzymes harboring biotechnological prospectives (Korndorfer *et al*., 2006).

Endolysins have applications in specific enrichment of microbial cells by their magnetic separation and immobilization. This novel application is based on affinity of Cell wall Binding Domains (CBDs) of phage encoded peptidoglycan hydrolases for host bacterial cell wall. Such polypeptide endolysins exclusively recognize the specific ligands on the gram-positive cell wall such as of *Bacillus cereus*, *L. monocytogenes* and *Clostridium perfringens* with high affinity. The CBD-based Magnetic Separation (CBD-MS) procedure has shown significant results when paramagnetic beads coated with recombinant Listeria phage endolysin-derived CBD molecules could capture and detect more than 90% of the viable *L. monocytogenes*; from artificially as well as naturally contaminated food samples that too within 20 to 40 min. Presence of other microorganisms in the same solution did not interfere the isolation procedure and needs less time, hence considered as superior to the already established traditional standard procedures. In general, endolysins such as CBD polypeptides signify modern pioneering tools for the capture of bacterial cells with promising relevance in microbial and diagnostics (Kretzer *et al*., 2007).

Neisseria gonorrhoeae encodes AtlA proteins with peptidoglycan transglycosylase homologus property, possessing peptidoglycan lytic activity similar to endolysins of bacteriophages (Kohler *et al*., 2007).

SENSITIVE TARGETS OF THERAPY

Endolysins act specifically against its target bacteria either in narrow range or in broad spectrum. Literature reveals examples of various bactericidal phage enzymes. Recombinant phage endolysins inhibit various pathogens and have recently been asserted as alternative antimicrobials for treatment of bacterial infections due to Gram-positive bacteria (Fischetti, 2003; Loessner, 2005). The effectiveness of phage lysins in clearing bacterial infections has been well documented in mouse models (Loeffler *et al*., 2001; Nelson *et al*., 2001; Schuch *et al*., 2002; Cheng *et al*., 2005; Rashel *et al*., 2007), in transgenic murine and bovine mammary glands (Kerr *et al*., 2001; Wall *et al*., 2005) and also in transgenic plants (De Vries *et al*., 1999). *Staphylococcus aureus*, Streptococcus uberis and *Steptococcus agalactiae* bacteriophage endolysins have been applied in mastitis cow's treatment (Xin *et al*., 1991; Donovan *et al*., 2006a, b; Celia *et al*., 2008) with profitable results.

Bacteriophage K1-5 encodes two different proteins originating from tail fibers capable of infecting K1 and K5 strains of *Escherichia coli* by replicating within it (Scholl *et al*., 2001; Kanamaru *et al*., 2005). Similarly, bacteriophage T4 tail lysozyme also acts as lysine enzymes. Bacteriophage phi3626 also produces murein hydrolase enzyme lysis system against many strains of *Clostridium perfringens* (Zimmer *et al*., 2002). *Staphylococcus aureus* and *Streptococcus agalactiae*, causal agent of mastitis mainly in high lactating cattle are also pathogenic for humans. To check the bacterial infection, *S. agalactiae* bacteriophage B30 induced two endolysins have been used. When these two novel antimicrobials of 182-amino-acid length, endolysins were allowed to fuse with the lysostaphin protein of *Staphylococcus simulans*, this fusions exhibited lytic activity for streptococcal as well as *S. aureus* pathogens. Immunohistochemical studies have shown that fusion proteins remain active in milk against bacteria with no harmful effects on the cells. It can successfully be used as an alternative to broad-range antibiotics against clinical infections since the fusion peptidoglycan hydrolase acts selectively as multi-pathogen targeting antimicrobial agent. One recombinant endolysin 11 is capable of hydrolyzing not only heat killed staphylococci but also staphylococcal biofilms. Another phage lysin LysK is a recombinant endolysin protein exerting lytic activity against clinically

relevant as well as methicillin-resistant *Staphylococcus aureus* (Navarre *et al*., 1999; O'Flaherty *et al*., 2005; Donovan *et al*., 2006c; Sass and Bierbaum, 2007). The endolysin LysH5 from the *Staphylococcus aureus* bacteriophage ΦH5 resembled other murein hydrolases encoded by staphylococcal phages. It rapidly lyses bovine and human *S. aureus* and human *Staphylococcus epidermidis* strains in pasteurized milk. Few other staphylococcal phage endolysins have been acquired from phages such as phi11, Twort, 187, P68, phiWMY and phage K (Xin *et al*., 1991; Loessner *et al*., 1998, 1999; O'Flaherty *et al*., 2005; Takac *et al*., 2005; Yokoi *et al*., 2005; Sass and Bierbaum, 2007). It signifies antimicrobial activity of a phage endolysin to be a part of novel biocontrol strategies in dairy industry.

Similarly, few endolysins from bacteriophages CMP1 and CN77 have also been used for biocontrol of plant-pathogen *Clavibacter michiganensis*. Against *C. michiganensis* subsp. *nebraskensis* and *C. michiganensis* subsp. *michiganensis*, His-tagged endolysin of CMP1 of 306 amino acids (34.8 kDa) and CN77 comprising of 290 amino acids (31.9 kDa), respectively, were cloned and expressed in *E. coli*. Both the enzymes in purified form are highly specific as they showed host specific bacteriolytic activity only against *Clavibacter* but not to any other closely related genera (Wittmann *et al*., 2010).

SOURCE OF ENZYBIOTICS

Many Ply endolysins are identified and isolated from variety of bacterial species, among them few are discussed here. Encoded by Ply genes three endolysin proteins from *Bacillus cereus* bacteriophage Bastille, TP21 and TP12 have also been produced in *E. coli*. These were isolated as recombinant proteins and purified by two step chromatography. All the three enzymes rapidly and specifically lyse several *Bacillus* species, with highest lytic activity against *B. cereus* and *B. thuringiensis*. Ply12 and Ply21 were chemically N-acetylmuramoyl-L-alanine amidases. Each of lytic enzymes (Ply Ba, 41.1 kDa; Ply21, 29.5 kDa, Ply12, 27.7 kDa) show significant heterogeneity in their amino acid sequence and molecular weight with only little similarity. Phage lysin proteins display that the catalytic/enzymatic activity is due to the N-terminal region which resembles with the cell wall hydrolase and autolysin (CwlSP, CwlA) of *B. subtilis*; while the C-termini of proteins are responsible for specific recognition and binding with the peptidoglycan of *Bacillus* spp. The close relationship of the phage lytic enzymes and cell wall autolysins reflects an indication towards horizontal gene transfer or sharing among various Bacillus phages and their hosts (Loessner *et al*., 1997; Porter *et al*., 2007).

Bacillus anthracis prophage Ba02 endolysin is another PlyL encoded by the *Bacillus anthracis* genome. PlyL is an N-acetylmuramoyl-L-alanine amidase capable of cleaving the cell wall of several *Bacillus* species when applied exogenously. It is observed that the catalytic domain of PlyL cleaves more efficiently than the full-length protein. Cell wall-binding domain showed strong binding to *B. cereus* comparative to other species like endolysin (Ply21) of *B. cereus* phage, TP21. Studies showed that the C-terminal domain sometimes inhibits the activity of the catalytic domain through intramolecular interactions but targeting of the enzyme to the cell wall externally is not a prerequisite of its lytic activity. These facts may be helpful while considering endolysins as therapeutic agents (Schuch *et al*., 2002; Low *et al*., 2005).

PlyC is a bacteriophage lysine containing two sub-units, PlyCA and PlyCB, which altogether exert murine hydrolase action against *Streptococcus pneumoniae* cell wall. This prevent colonization of group A streptococci in the upper respiratory tract of mice and leads to bacterial exclusion by killing the microorganism (Loeffler *et al*., 2001; Nelson *et al*., 2001, 2006).

Listeria monocytogenes bacteriophage also encode lytic endolysin enzymes which harbors specifically hydrolyzing cross-linking peptide bridges for Listeria peptidoglycan. Two endolysins, Ply118, a 30.8 kDa L-alanoyl-D-glutamate peptidase and Ply511, a 36.5 kDa N-acetylmuramoyl-L-alanine amidase, have been used with the aim of biopreservation properties against *L. monocytogenes* in the food, specifically in dairy starter cultures. Endolysins ply118 and ply511 are used for production of lytic enzyme by genetic fusion with *Lactococcus lactis* MG1363. Therefore, ply511 was fused with the $_{SP}$slpA nucleotide sequence encoding the Lactobacillus S-layer protein signal peptide. Expression of $_{SP}$slpA-ply511 from pSL-PL511 resulted in secretion of functional ply511 enzyme from *L. lactis* cells which showed unusually strong lytic activity due to frame shift mutation occurred in final secretory product. Surprisingly, the resulting mutant polypeptide strongly increased its lytic activity. Immunoblotting experiments indicated that the enzyme caused rapid lysis of *L. monocytogenes* cells (Loessner *et al*., 1995, 2002; Gaeng *et al*., 2000).

Mur-LH is a broad-spectrum endolysin obtained from temperate bacteriophage Φ-0303 of *Lactobacillus helveticus* CNRZ 303 strain. The lysin-encoding lys gene of this bacteriophage was cloned using a library of Φ-0303 in *E. coli* DH5a. The lys gene sequence has 1,122 bp encoding a protein of 373 amino acids (Mur-LH), with lytic activity and molecular mass of 40.2 KDa. Mur-LH endolysins was expressed in *E. coli* BL21, its N-terminal sequence showed catalytic activity and caused hydrolysis of *L. helveticus* CNRZ 303 cell walls. Endolysin Mur-LH possesses N-acetylmuramidase activity which provides broad spectrum of lytic activity against different species such as *Bacillus subtilis*, thermophilic lactobacilli and lactococci, pediococci, *Brevibacterium linens* and *Enterococcus faecium*. Many other lytic endolysin enzymes with broad lethal activity have been identified even against antibiotic-resistant *Enterococcus faecalis* and *Enterococcus faecium* bacteria (Deutsch *et al*., 2004; Yoong *et al*., 2004).

LysA, a 303 amino acid protein, has up to 35% identity with endolysins from prophages Lj928 and Lj965 from *Lactobacillus johnsonii* and Lp1 and Lp2 from *Lactobacillus plantarum* as well as with the endolysin of *Lactobacillus gasseri* bacteriophage Fadh. The N-terminus of LysA has N-acetylmuramidase catalytic activity while the C-terminus has sequence similarity with putative cell envelope binding bacterial SH3b domains present in the majority of Lactobacillus bacteriophage endolysins. LysA protein expressed in *E. coli* cells demonstrated wide range of bacteriolytic activity against several members of Lactobacillus species, *Lactococcus lactis*, streptococci and *Staphylococcus aureus*. It is evident that LysA is 2 and 8000 times more active against *L. fermentum* (lytic enzyme BR11) than *L. lactis* and *Streptococcus pyogenes* bacteria, respectively (Sugahara *et al*., 2007; Turner *et al*., 2004, 2007).

MAJOR CLASSES OF ENZYBIOTICS

Bacteriocins: Typically, bacteriocins are considered as antibiotics (narrow spectrum) and are bacterial toxins that are proteinaceous in nature. On the basis of phenomenological relation there are various large categories of bacteriocins which include those from gram positive bacteria (the colicins) along with those from Archea known as the microcins (Cotter *et al*., 2006; Cascales *et al*., 2007). They cause inhibition of growth of strains of bacteria that are either similar or closely related (Farkas-Himsley, 1980). Non-pathogenic bacteria produce bacteriocins that use to colonize the body of human normally. The loss of bacteriocin producing harmless bacteria may lead to invasion of the body by opportunistic pathogens (Cruz-Chamorro *et al*., 2006; Sand *et al*., 2007).

Lysins: Lysins otherwise known as endolysins are basic enzymes cleaving peptidoglycans' covalent bond and are encoded by bacteriophages having double stranded Deoxy Ribonucleic Acid (DNA) (Borysowski *et al*., 2006; Fischetti, 2010). At pH lower than isoelectric point they are basic enzymes having positive charge (Mullan, 2003). On the basis of the specific sites the enzyme acts, lysins are grouped into five major classes: N-acetylmuramoyl-L-alanine amidases, endopeptidases, N-acetylmuramidases (lysozymes), endo-β-N-acetylglucosaminidases and lytic transglycosylases. Peptidoglycan degrading proteins are the endolysins having a characteristic lytic structure often with domains having multiple lytic or cell wall binding domains. A range of new applications have been opened by endolysin engineering for the proteins from safety of food to decontamination of environment to antimicrobials that are more effective and are believed refractory to development of resistance (Nelson *et al*., 2012). Access to the peptidogycan and subsequent destruction of the bacteria bearing them is provided by endolysin because of absence of an outer membrane in the cell wall of gram positive bacteria. For optimization of endolysins for useful applications, specifically molecular engineering techniques can be used. In the field of medicine and food safety, agriculture and biotechnology, new development of lysins are the latest upcoming issues (Schmelcher *et al*., 2012).

Lysozymes: Catalysis of 1, 4-beta-linkage hydrolysis between N-acetylmuramic acid and N-acetyl, D-glucosamine is done by lysozyme by damaging the cell wall of bacteria. Lysozyme is abundant in a number of secretions and especially in the egg white large amount of lysozyme enzyme has been found. For gram positive pathogens like *Bacillus* and *Streptococcus* a natural form of protection is provided by lysozyme and thus it is considered as natural antibiotic. It is also an integral component of innate immunity and considered as a unique enzybiotic in that it exerts both the antibacterial as well as anti-viral and anti-inflammatory activities (Sava, 1996; Helal *et al*., 2012).

Autolysin: Autolysin is an enzyme hydrolyzing a biological cell or tissue's component in which it is produced. It is similar in function to a lysozyme. Cleavage of the β-(1, 4) bond between N-acetylmuramic acid and N acetyl glucosamine is brought about by autolysin. Regulation of autolysin by gram positive bacteria is brought about by molecules of teichoic acid that is attached to the tetra peptide of the matrix of the peptidoglycan (Smith *et al*., 2000). Atl is the major autolysin of *Staphylococcus epidermidis* and *S. aureus* playing an important role in separation of cell and in virulence, their virulence are also attenuated. For the development of new types of antibiotics autolysins represent a promising target (Zoll *et al*., 2010). For the pathogenesis of infections that are invasive in nature presence of pneumolysin appears to be more critical. In case of all isolates of *Streptococus pneumoniae* presence of lytA gene signifies that autolysin is an obligate necessity for this organism irrespective of the isolation site (Qin *et al*., 2007; Sourav *et al*., 2010).

Other potential enzybiotics: The defensins as well as cathelicidins can be exploited as enzybiotics among other families of antimicrobial peptide gene. In the innate immunity such antibiotic peptides that are endogenous in nature play crucial role and forms the first line of defense for protecting the internal as well as the external body surface of the host (Wang *et al*., 2011). Bacterial viruses or bacteriophages are source of several enzymes used in enzybiotics besides plants even though use of other natural or synthetic agents can be in vogue (Haq *et al*., 2012). For killing the bacterial cells, lysins derived from bacteriophages can be incorporated through the enzybiotic approach (Hermoso *et al*., 2007). When used in combination with the antibiotic

gentamicin, antimicrobial activity can be exhibited by lysine that is isolated from P68 phage of *Staphylococcus aureus* (Manoharadas *et al*., 2009).

BENEFICIAL APPLICATIONS

Role of bacteriocin in food industry: There has been incorporation of nisin as well as lacticin commercially as prophylactic measures against mastitis. As an alternative to antibiotic, feeding of bacteriocins has also been suggested. There has also been reduction in the carriage of zoonotic pathogens as the producers of bactericins have got the capability to colonize in the gastrointestinal tract (Diez-Gonzalez, 2007; Line *et al*., 2008).

Role of endolysins in food industry: Bacteriophage encoded endolysins have been recently deemed as new emerging biocontrol tools to inhibit and check the food contamination by pathogens in food industry. The ionic concentration plays an important role for optimal lytic activity of lysins such as LysH5, the endolysin encoded by the staphylococcal bacteriophage phi-SauS-IPLA88. Ca^{++}, Mg^{++} and NaCl enhanced the activity of LysH5. The activity was inhibited by the presence of Mn^{++} and Zn^{++}. Along with another food biopreservative, bacteriocin nisin LysH5a portray strong synergistic effect specifically against *S. aureus*. Such study paves the way to exploit the possibilities of hurdle technology, part of Hazard Analysis and Critical Control Point (HACCP) combining a phage-encoded endolysin and the bacteriocin nisin for efficient *S. aureus* inhibition in milk and other dairy products (Brussow, 2001; Garcia *et al*., 2010). Currentlly, two commercial bacteriophage cocktails that encounter Listeriosis, Listex_P100 (Micreos) and ListShield (Intralytix), have received approval from Food and Drug Act (FDA) (Loessner, 2005; Shuren, 2006).

Use of enzybiotics in farm animals: Emerging resistance to antibiotics along with the threat of antibiotic residues have got negative influence on human health. Both European Union and United States have prohibited the usage of antibiotic in this context (Shinde and Chae, 2009). Certain strains of *Lactococcus lactis* subsp. *lactis* produce enzybiotic nisin which can be used in foods as well as poultry products. For developing bacteriocins and antimicrobial peptides; bacteriophages research is in progress further (Joerger, 2003). Putative lysine is a new antimicrobial growth promoting agent which is suggested by the European Union. It is found to be a better alternative than prebiotics and probiotics; as well as phytonutrients and hyperimmune antibodies (Seal, 2013). In the intrapartum prophylaxis in case of early onset of neonatal infections due to *Streptococcus agalactiae* that colonizes the genital tract the major potential application for the lytic enzymes have been proposed (Pritchard *et al*., 2004; Cheng *et al*., 2005). PlyGBS is the only lysine specific to *S. agalactiae* whose efficacy has been evaluated *in vivo* (Cheng *et al*., 2005). In case of colonization in the vagina in murine model one topical dose of lysine when administered has resulted in 3-log decrease in the level of bacteria in comparison to mice in control group. One such dose of PlyGBS topically is also sufficient to cause reduction in colonization of bacteria of the mucosa of oropharynx sufficiently. It thus appears that lytic enzymes specific to *S. agalactiae* may be used not only for the elimination of colonization in vagina before delivery in case of pregnant animals but also for decontamination of new borns; thereby causing decrease in the incidence of infections in neonates. In a recombinant form administration of these enzymes can be done topically or can be secreted in the genital tract by bacteria that are engineered (Borysowski and Gorski, 2010). There has also been report of the potential of bacteriocin as antimicrobial agent as well as growth promoter in the animal disease treatment. Demonstration of the characterization of a bacteriocin produced by a LFB112 strain of *Bacillus subtilis* that has been isolated from

Chinese herbs has been reported in this context (Cole *et al*., 2006; Xie *et al*., 2009). In the prophylaxis as well as treatment of several bacterial infections that include pharyngitis and tonsillitis, dysentery as well as infections caused by wound, there has been use of lysozymes in combination with other antibiotics for the last several decades (Sava, 1996). There have been patents either applied for or issued for various lysozymes more recently (Donovan, 2007). These include the use of formulation of lysozyme either as a gel for treatment topically in case of wounds; acne's treatment by using several formulations of the enzyme; and infection prophylaxis due to piercing of skin. This also includes the use of lysozyme that is aerosolized for treating tracheitis and pneumonia; amyglalitis as well as faucitis. The use of mutants of lysozyme for neutralizing the activity of a lysozyme inhibitor produced by *Treponema pallidum* is another interesting application of lysozyme. As a component of oral health products including mouthwashes, lysozyme has also been used for the purpose of killing several bacteria in the oral cavity (Tenovuo, 2002; Gil-Montoya *et al*., 2008). It has been shown in a recent study that utilization of lysozyme can be done as a carrier that allows delivery of antibiotic molecules specifically to bacterial cells (Hoq *et al*., 2008).

Among the endopeptidase, lysostaphin is a major one having potential therapeutic application. The foremost medical application of lysostaphin is Staphylococci elimination that colonizes the membrane of the nasal mucosa. This in certain clinical settings may be a starting point of infections that are serious. In a cotton rat model of *Staphylococcus aureus* nasal colonization the efficacy of lysostaphin has been shown more than mupirocin which is at present the main antibiotic used as a decolonizing agent (Kokai-Kun *et al*., 2003). Preventing colonization of catheter by molecules of enzymes coating their surface is another prophylactic use of lysostaphin (Shah *et al*., 2004). Both topical as well as systemic application of lysostaphin in Staphylococcal infections is its second major application. In experimental models of bacteremia and endocarditis, as well as neonatal and ocular infections (especially in case of endophthalmitis and keratitis), evaluation regarding the therapeutic effectiveness of lysostaphin has been done (Patron *et al*., 1999; Dajcs *et al*., 2001; Kokai-Kun *et al*., 2007; Oluola *et al*., 2007). It has been revealed by such studies that lysostaphin can kill bacteria *in vivo* efficiently without causing any side effect that may prove serious. It is quiet noteworthy that in certain experiments the efficacy of lysostaphin is found to be more than antibiotics (Climo *et al*., 1998). The enzyme can exert antibacterial activity substantially even after injection repeatedly (Dajcs *et al*., 2002). A synergistic antibacterial activity with other lytic enzymes, antimicrobial peptides (cationic) along with certain antibiotics is also possessed by lysostaphin (Becker *et al*., 2008).

Homeostasis of the body: The results of the preclinical studies indicate that certain dilemma such as immunogenicity, the release of pro-inflammatory components during bacteriolysis, or the development of resistance associated with endolysin therapy may not seriously affect their use in regards to the safety and therapeutic effectiveness of endolysins.

COMPARISON WITH OTHER EMERGING ALTERNATIVE BIOLOGICAL THERAPIES

Several other contemporary alternative therapies are also evolving in parallel to Enzybiotic therapy, these are discussed in brief as below.

Bacteriophage therapy: Bacteriophages are viruses of bacteria which invade their host bacterium by using specific receptors but do not affect eukaryotic cells being host specific. In their

lytic mode of life-cycle, by secreting endolysins and holin enzymes phages can kill Gram positive, Gram negative, Acid-fast and many other bacteria as well. Bacteriophage therapy has been tried for a wide range of bacterial infections for animals and humans (Hankin, 1896; Twort, 1915; Clark and March, 2006; Tiwari *et al*., 2011; Ghannad and Mohammadi, 2012; Dhama *et al*., 2013c; Tiwari *et al*., 2012, 2014a).

Virophages and mycophages: These are viruses which act specifically against viruses and fungi, respectively. Virophage and mycophage therapies are the new emerging concepts, as antiviral drugs and antifungal drugs require long-term medications and may have many side effects. The mycophages and virophages can be modified and used in therapeutic preparations for the treatment of diseases against many pathogenic fungi and certain viruses and can thus reduce antifungal and anti-viral resistance to a certain extent (Ghabrial, 1980; Skurnik and Strauch, 2006; Koonin, 2012; Tiwari *et al*., 2014b).

Cytokine therapy: Cytokines are intercellular regulatory proteins, which play a pivotal role in initiation, maintenance and regulation of immunological homeostatic and inflammatory processes. Due to their multiple functions, they are promising candidates for therapeutic interference in infectious and autoimmune diseases, especially in immunosuppressed patients receiving long term treatment for cancer or Acquired Immunodeficiency Syndrome (AIDS). The immunoglobulin Fc fragment based cytokines provides superior therapeutic approach. Nevertheless, the development of new vaccines necessitates the development of new types of cytokine adjuvants to ensure an appropriate immune response (Antachopoulos and Roilides, 2005; Jazayeri and Carroll, 2008; Nicholls *et al*., 2010; Dhama *et al*., 2013d).

Avian egg antibodies therapy: Chicken are capable of producing antigen specific antibodies (IgY), which have function similar to IgG in response to antigen. It can be used to treat microbes, which do not respond to antibiotics. Treatment with these antibodies produced in eggs of hyperimmune birds is safer, more efficient and less expensive in comparison to antibiotics. Specific IgY antibodies have been developed against different bacterial or viral pathogens viz., rotavirus, bovine respiratory syncitial virus, coronavirus, infectious bursal disease virus, *E. coli, Salmonella, Edwardsiella, Yersinia, Staphylococcus, Streptococcus* and *Pseudomonas* (Yegani and Korver, 2007; Rahimi *et al*., 2007; Michael *et al*., 2010; Da Silva and Tambourgi, 2010; Dhama *et al*., 2011, 2013c; Ferella *et al*., 2012).

Herbal therapy: Various herbs and their extracts have been proved to have potent antimicrobial, antiviral or antifungal activities (Fabricant and Farnsworth, 2001; Cravotto *et al*., 2010; Hashemi and Davoodi, 2012; Mahima *et al*., 2012, 2013a; Dhama *et al*., 2013c; Tiwari *et al*., 2013b). For example, neem, ashwagandha, giloy, onion, garlic, mustard, red chili, turmeric, clove, cinnamon, saffron, curry leaf, fenugreek, ginger etc., have been found to be highly useful in this aspect. Also, herbs do not possess development of resistance like that of antibiotics and are also comparatively safer and cost-effective. Globally, researches are exploring the potential role of plants and their extracts in enhancing the immunity of man and animals and thereby encouraging avoidance of antibiotics. Herbal therapy is also gaining much attention these days in the treatment of subclinical mastitis and uses of *Terminalia chebula* and *Terminalia belerica* in this regard are found to be significant (Hawari and Al-Dabbas, 2008; Deb *et al*., 2013).

Panchgavya therapy: Nowadays, Panchgavya therapy (cowpathy) is also gaining much importance because cow urine (an important component of Panchgavya) is able to kill a number of bacteria that show antibiotic resistance. The antibiotic resistance germs of tuberculosis can be killed by cow dung and urine, particularly cow urine acts as a bioenhancer for anti-tuberculous drugs, for which it is gaining much importance in the international market as an anti-tubercular agent (Dhama *et al*., 2005, 2013e).

INFORMATION PORTAL OF ENZYBIOTICS

EnzyBase: A comprehensive as well as web-accessible database of enzybiotics is EnzyBase that may aid to the enhancement of our understanding of enzybiotics at present along with their mechanisms of action and new drug development for application in medical science. It has got a diverse potential application that include: Cocktails as well as designing of novel enzybiotic which is beneficial in response to emerging pathogens that are resistant to drugs continuously. Exactly 1144 enzybiotics along with 216 natural resources have been included in the current version of EnzyBase (Skurnik and Strauch, 2006; Wu *et al*., 2012). The top rated sources of enzybiotics include *Staphylococcus aureus* that is infected with phage; *Enterococcus faecalis*, *Bacillus cereus*, *Streptococcus pneumonia*, *Bacillus thuringiensis*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, *Clostridium perfringens* and *Enterococcus faecium*. The narrow spectrum of antibacterial activity is one of the weaknesses of enzybiotics. But against a wide variety of bacterial infections along with their resistant strains, enzybiotics in combination with several spectra of antibacterial activities and various mechanisms of actions can be used much beneficially. It is easier to use the interface of EnzyBase allowing users to retrieve rapidly data in accordance to their desired criteria of search along with blasting of the database for sequences that are homologous (Ahluwalia and Sekhon, 2012).

phiBIOTICS: Unique therapeutic capabilities of enzybiotics have been confirmed by numerous experimental studies thereby increasing the attention of the medical community in wider sense. For summarizing the state of knowledge of enzybiotics, currently phiBIOTICS has been developed which is an information portal about therapeutic enzybiotics that are known and studied. Informations regarding chemical as well as biological properties of enzybiotics together with compendium of facts that are retrieved from research studies are contained in the phiBIOTICS. For predicting the novel potential enzybiotics phiBiScan program utility is dedicated (Hojckova *et al*., 2013).

CONCLUSION AND FUTURE PERSPECTIVES

This review discusses the prophylactic and therapeutic applications of endolysins, especially with respect to their potential use in human and animal medicine. Due to increase in the prevalence of multidrug resistant bacteria dramatically and continuously, the most crucial characteristic of enzybiotics is their mode of action which is novel along with the ability to combat bacteria that are resistant to antibiotics. In relation to traditional antibiotics the risk of development of resistance is relatively lower for certain lytic enzymes. Enzybiotics that are unmodified importantly lyse solely gram positive bacteria but certain modifications that are developed enable them to kill gram negative bacteria as well. Various forms of prophylaxis as well as treatment of bacterial infections are included in the potential medical applications of enzybiotics. In animal models for instance certain lytic enzymes have been shown to be very effective in killing bacteria

that colonize mucous membranes upon administration topically. Employment of such enzymes can be done as unique means of prophylaxis on the basis of clearance of bacteria that represents a starting point for infections potentially. In the treatment of several systemic infections (including bacteremia) in animals that are immunized too it has been shown by various experimental studies that lytic enzymes are efficacious. Being most abundant biological entities on earth phages are a rich natural source of endolysin enzymes, hence with enormous potentials Lysins can be explored against infectious disease even in the dilemma of multi-drug-resistance conditions also. Further digging will definitely lead to produce new opportunities for the production of specifically engineered designer lysins with diverse applications in biology and life sciences for the wellbeing of humanity against deadly pathogens and infections. On the basis of the unique therapeutic capabilities, enzybiotics certainly deserve attention in the wider sense of the medical community.

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