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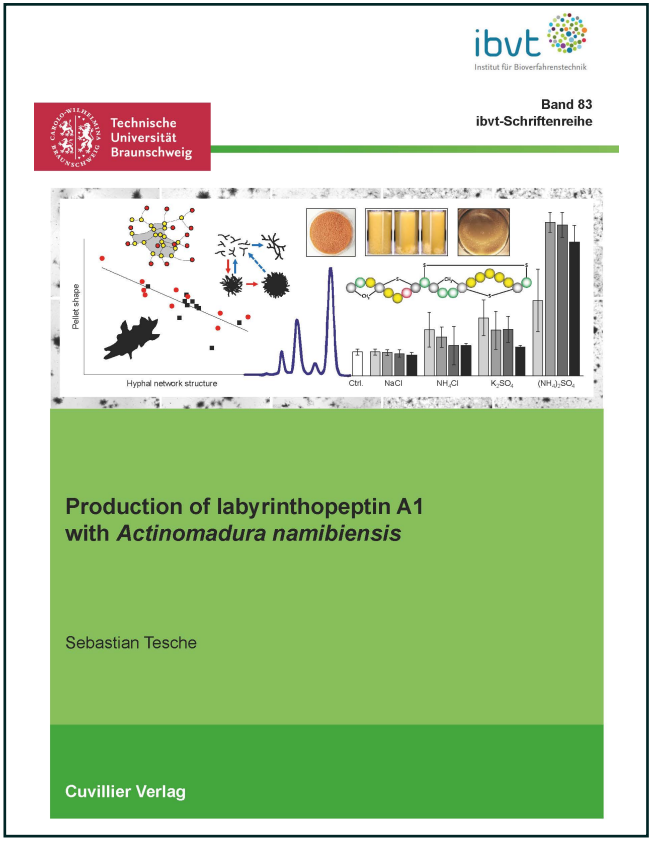
Production of labyrinthopeptin A1 with Actinomadura namibiensis



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1 Introduction and objectives

For decades now, filamentously growing actinomycetes have been one of the most important sources for the discovery of antimicrobial active ingredients [1]. With the proliferation of a high number of antibiotic compounds, however, pathogenic strains have become increasingly resistant. Today, antibiotic and also antiviral resistance has reached a critical point, so that there is a great need for novel agents [2, 3]. In this regard, small peptides drugs have attracted attention of the pharmaceutical industry [4]. One of the most promising new drug candidates for the treatment of various diseases are lantibiotics, which are ribosomally synthesized and post-translationally modified peptides (RiPPs) produced by Gram-positive bacteria [5]. The potency of lantibiotics against multi-drug resistant strains originates from their unique structure with unusual amino acids, which is associated with distinctly different mechanisms of action. With their size of 19 to 38 amino acids, lantibiotics may combine the advantages of small molecules and protein drugs [2].

Labyrinthopeptins are an interesting subgroup of lantibiotics and display highly attractive bioactive properties. The only known producer of these molecules is an actinomycete which was isolated in 1988 as part of a project of Hoechst AG (today: Sanofi-Aventis) from a soil sample of the Namib Desert, Namibia. In early screenings, a weak antiviral activity against several strains was found. But the project has not been followed up since the structural elucidation of the active compounds was too difficult [6]. It was not until 2003 that the producing organism was described and identified as the novel species *Actinomadura namibiensis* [7]. Some years later the peptide structure of the bioactive compounds could be solved and was patented before publishing it in 2010 [8, 9]. Three variants, labyrinthopeptin A1, A2 and A3, were found in the culture filtrates of *A. namibiensis*. Meanwhile, further studies on the biological efficacy were conducted. Labyrinthopeptin A1 demonstrated antiviral activity against human immunodeficiency virus (HIV) and herpes simplex virus (HSV) at low concentrations without toxic effects on vaginal lactobacilli and without the inflammatory response of peripheral blood mononuclear cells. Therefore, it is of special interest for the treatment of sexually transmitted viruses [10]. In addition, the peptide displayed activity against human respiratory syncytial virus subtypes A and B (hRSV) [11]. Moreover, synergistic effects with other standard antiretroviral drugs [10] were found, making labyrinthopeptin A1 a promising candidate for the development of a broad-spectrum antibiotic as well [12]. By contrast, Labyrinthopeptin A2 showed an antiallodynic activity (against neuropathic pain) [9], and labyrintopeptin A3 was identified as a degradation product of labyrinthopeptin A1 [8].

The broad spectrum of biological activity of labyrinthopeptin is responsible that research on the production methods recently intensified. First steps towards the chemical synthesis of labyrinthopeptins have been made [13]. However, total synthesis of labyrinthopeptins is not yet possible, and the economic feasibility is questionable [14]. Thus, biotechnological production using the wild-type [15] or another filamentous production host [16] remains the most prospective method. In 2018 first results of a production process for labyrinthopeptin A1 and A2 with *A. namibiensis* in 10-L scale bioreactors and a downstream method to purify labyrinthopeptin A1 and A2 were published [15]. Nevertheless, the product concentration of the most interesting drug candidate labyrinthopeptin A1 that was achieved with the wild-type or transgenic hosts remained at low concentrations in the range of 80 to 150 mg L^{-1} [15, 16].

Cultivation of filamentous organisms is particularly challenging. Their growth in submerged culture depends on many factors, such as the medium composition and pH value, the method of inoculation and the general operation conditions of the cultivation process (temperature, shaking or stirring rate and the resulting fluid-dynamic stress, oxygen transfer, etc.). All these factors can affect the organisms' cellular morphology, which in turn has an influence on the growth rate and – most importantly – the productivity for the desired product [17]. The cellular morphology of filamentous organisms shows a high variability and may range from dispersed hyphae to very dense agglomerates of hyphae, the so-called pellets. If the culture exhibits a predominantly dispersed growth of hyphae, the apparent viscosity of the cultivation broth increases drastically, which is associated with special demands on the power input to avoid a reduction in mass transfer rates and concentration gradients of nutrients and oxygen [18]. By contrast, if the hyphae tend to form pellets, an insufficient supply with nutrients and oxygen of the inner part of the pellets will eventually occur as they increase in size [19]. Theoretically, small, hairy pellets would be the perfect tradeoff between the advantages and disadvantages of dispersed and pellet morphology. However, it must always be considered that, depending on the organism and the desired product, different morphologies are favored for high product yields [20–24]. Therefore, it is necessary to find process parameters that provide the optimal balance between homogeneous substrate and oxygen distribution in the cultivation broth and the favored mycelial structures in terms of productivity.

Knowing that cell morphology of filamentous microorganisms has a major impact on the productivity, several methods to tailor morphologies for enhanced yield of a desired product have been developed. For instance, the morphology can be influenced by the choice of nutrient sources, the input of physiological stress (e.g., by microparticle-enhanced cultivation (MPEC) [25–28]) or by other additives [29–31]. A method that has hardly been used so far is the manipulation of the medium osmolality by the addition of inorganic salts to the cultivation

broth. Since osmoregulation is inseparable from metabolic regulation [32] and metabolic regulation affects morphology and productivity, the addition of salts to the cultivation medium can be used to alter the morphology. In this thesis, this method was chosen to adjust the morphology of *A. namibiensis* towards higher productivity of labyrinthopeptin A1.

The development and time-dependent change of the cellular morphology is usually quantified by image analysis of microscopic pictures. As mycelia give structure to the culture suspension, the biomass concentration and the distribution of size and shape of the cells are also linked to the flow properties of the cultivation broth. Thus, the knowledge of basic rheological properties is essential for understanding the cultivation of filamentous microorganisms. Moreover, rheological measurements can be used to follow the condition of the culture, especially with regard to the average solidity of the hyphal network. In the present thesis key parameters from rheological measurements are correlated with morphological parameters obtained from image analysis to understand the connection between rheology and morphology in cultivations of *A. namibiensis*.

After cultivation, the desired product needs to be recovered and purified (downstream processing). Therefore, a crude extract from the cultivation broth is made first, usually by solvent extraction, adsorption, ultrafiltration or precipitation. The subsequent purification process is a critical step during the production of (bio)pharmaceuticals since it accounts for up to 80 % of the total production costs [33, 34]. For protein purification mainly chromatography methods are applied. The suitability of chromatographic methods to separate the labyrinthopeptin derivatives was also investigated in the framework of this thesis.

The primary aim of this work was to increase the labyrinthopeptin A1 yield in the cultivation of *A. namibiensis*. In order to gain a better understanding of the influence of various cultivation parameters on the shaking flask cultivation and productivity of this poorly described organism, the following objectives were pursued:

- Optimization of the shaking flask cultivation process and characterization of the growth and product formation mechanisms.
- Generation of a yield-enhancing morphology through manipulation of the cultivation medium osmolality, and quantification of the morphological changes by image analysis of microscopic pictures.
- Correlation of the rheological properties of the cultivation broth with its morphology.
- Recovering of the labyrinthopeptins from cultivation broth and removal of impurities by chromatography.

2 Theoretical background

2.1 Characteristics of labyrinthopeptins

In 1988 the Gram-positive, filamentous bacterium *Actinomadura namibiensis* (type strain HAG 010767T / DSM 44197T) was isolated from the Namib Desert. It was first described by Wink et al. [7] in 2003 and belongs to the class of acintomycetes [7, 35]. Depending on the cultivation medium, the strain develops characteristic white aerial mycelium and salmon red substrate mycelium. In the aerial mycelium, the formation of spiral spore-chains was observed [7]. From culture filtrates of *A. namibiensis* labyrinthopeptins were isolated for the first time and their structure was finally determined in 2010 [9]. Until now, *A. namibiensis* is the only known natural producer of labyrinthopeptins.

2.2.1 Biosynthesis and structure

Recently, labyrinthopeptins came into focus as very promising new drug candidates. These molecules belong to the **ri**bosomally synthesized and **p**osttranslationally modified **p**eptide**s** (RiPPs), a diverse class of natural products of ribosomal origin. They are part of the sub-group of type III lantipeptides, which are polycyclic RiPPs with a size of < 5 kDa containing the non-canonical amino acids **lan**thionin (Lan) or β-**me**thyl **lan**thionin (MeLan) and dehydrated residues as characteristic structural elements [36–38]. Lan and MeLan are formally alanine and 2-aminobutyric acid, respectively, connected to another alanine at their β-carbon atoms via a thioether bridge (**Fig. 2-1**). Many lantipeptides, such as labyrinthopeptins, show antimicrobial activity and are therefore also called lantibiotics.

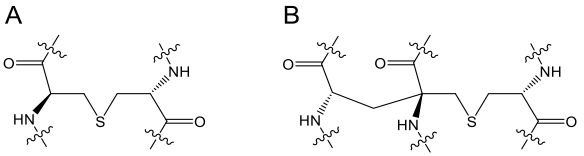

Figure 2-1: Structures of the amino acids A) lanthionin, characteristic of lantibiotics and B) labionin, characteristic of labyrinthopeptins.

In culture filtrates of *A. namibiensis*, three labyrinthopeptin derivatives, labyrinthopeptin A1, A2 and A3 (**Fig. 2-2**) were found [8]. Labyrinthopeptin A1 showed very effective *in vitro* antiviral activity against human immunodeficiency virus (HIV) and herpes simplex virus (HSV) – even against drug resistant strains – at submicromolar concentrations without toxic effects on vaginal lactobacilli and without the inflammatory response of peripheral blood

mononuclear cells; therefore, it has a great potential for the treatment of sexually transmitted viruses [10]. Furthermore, activity against human respiratory syncytial virus (hRSV) [11] and synergistic effects with other standard antiretroviral drugs was proven [10], making it a candidate for the development of a broad-spectrum antibiotic as well [12]. Labyrinthopeptin A2 displayed only moderate activity against HSV and no anti-HIV activity but an activity against neuropathic pain in a spared nerve injury mouse model [9]. Labyrintopeptin A3 is a degradation product of labyrinthopeptin A1 [8].

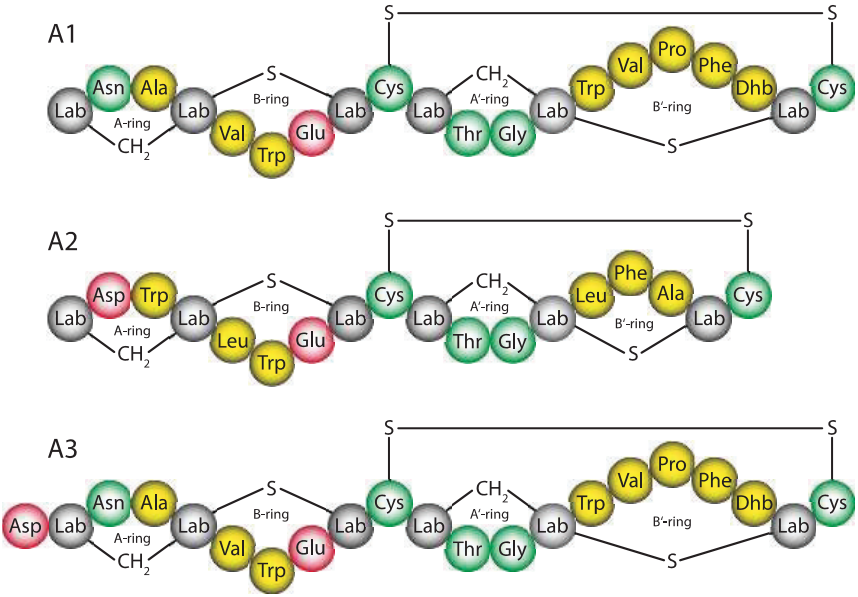

Figure 2-2: Structures of labyrinthopeptin A1, A2 and A3. Colors of the amino acids illustrate the physicochemical properties of their side chains: polar (green), hydrophobic (yellow) and acid (red). Modified from [9] (abbreviations according to IUPAC nomenclature [39], Dhb = didehydrobutyrine, Lab = labionin).

The general biosynthesis of lantibiotics commences with the translation of a linear propeptide (LanA) consisting of a leader peptide and a core peptide, which is modified by at least one enzyme [23]. A special characteristic of lantibiotics is the formation of intramolecular ring structures [20, 24] that stabilize these molecules [25]. Subsequently, the leader peptide is removed by a protease and the mature lantipeptide is exported from the producing cell. All genes of enzymes that are involved in the maturation process of the peptide are located in a gene cluster [23].

Depending on the enzymes that are used to produce the peptide, lantibiotics are categorized in sub-types I to IV [40]. Labyrinthopeptins fall into class III but, unlike most other lantibiotics, they contain the unprecedented carbocyclic triaminoacid labionin (Lab), which is practically lanthionin extended by a methylene bridge to another amino acid. In several enzymatic reaction steps labionin is formed in the prepropeptide by linkage of the serine and cysteine residues of the underlying Ser-Xxx-Xxx-Ser-Xxx-Xxx-Xxx-Cys motif [9] (**Fig. 2-3**). This linkage also leads to the formation of two rings (named A and B) within the molecule, which are connected through a central quaternary C atom.

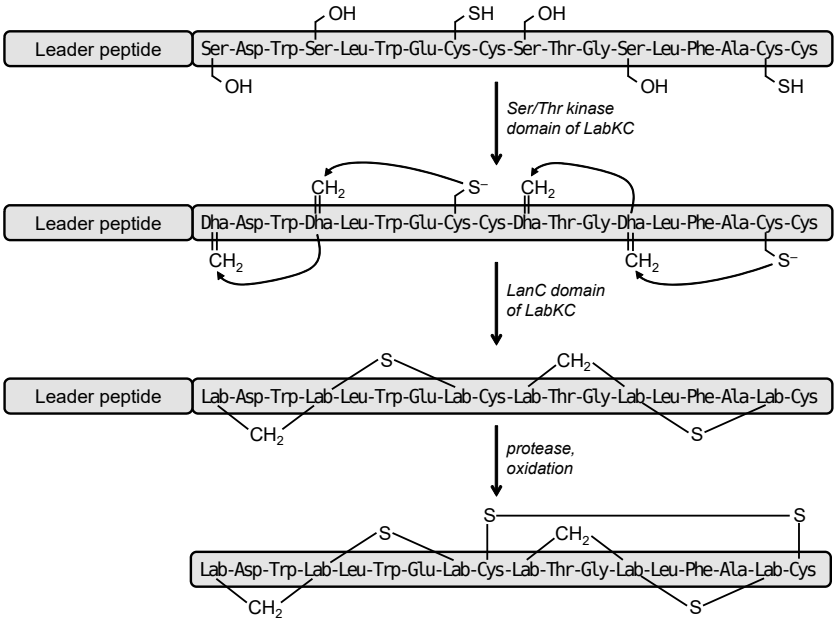

Figure 2-3: Scheme of the biosynthesis of labyrinthopeptin A2. The two-domain enzyme LabKC catalyzes phosphorylation (not shown), dehydration, and cyclization of the prepropeptide. Finally, remaining cysteine residues are oxidized and the leader peptide is cleaved off proteolytically [41] (abbreviations according to IUPAC nomenclature [39], Dha = didehydroalanine, Lab = labionin).

The labyrinthopeptin biosynthesis gene cluster lies in a 6.4 kb DNA sequence and includes five genes. The propeptide with its leader sequence is translated from the structural genes labA1/A3 and labA2. The genes labT1 and labT2 code for ATP-dependent transporter proteins to export the peptide from the cell and LabKC codes for a trifunctional synthetase with lyase, kinase and putative cyclase domains for the labyrinthopeptin synthesis [9, 40].

All three labyrinthopeptins mainly consist of unpolar amino acids. The glutamic acid might give the molecules a slightly acidic character. Labyrinthopeptin A1 and A2 both contain two labionins and a disulfide bond between a C-terminal cysteine and a cysteine at position 9 in the amino acid sequence. This leads to the formation of five rings in total and results in a globular structure of the mature peptide.

2.2.2 Production methods

For many lantibiotics a chemical synthesis is possible [14]. The complex stereo configuration of labionin, however, could not be achieved by chemical synthesis so far [13]. Generation of labyrinthopeptins by genetic engineering was investigated by Krawczyk et al. [16] in 250-mL shaking flasks with a 50 mL culture volume. The authors found the natural producer *A. namibiensis* to be genetically intractable; thus, heterologous expression of labyrinthopeptin analogues was performed with *Streptomyces lividans*. However, the labyrinthopeptin variants were not correctly processed and contained additional N-terminal amino acids. The production of the undesired labyrinthopeptins could be avoided by the construction of a synthetic labyrinthopeptin A1 gene with additional methionine at the -1 position of the leader peptide. Although transformation of the new synthetic gene resulted in the production of different labyrinthopeptin A1 variants as well, all of them were converted into labyrinthopeptin A1 after longer cultivation times. After 16 days of cultivation, a labyrinthopeptin A1 concentration of 86 mg L^{-1} [16] and a space-time yield (STY), often also referred to as volumetric product formation rate, of 5.4 mg L^{-1} d^{-1} was finally achieved. But the process was never scaled-up, and since the benefit of heterologous expression of labyrinthopeptins is uncertain, the production in the wild-type producer *A. namibiensis* is still important [42].

Rupcic et al. [15] showed the first results of a production of labyrinthopeptin at larger scale. From a 7.5-L cultivation broth of *A. namibiensis* 580 mg of labyrinthopeptin A1 and 510 mg of labyrinthopeptin A2 were isolated with recovery of 72.5 and 42.3 %, respectively. However, the cultivation, which resulted in a maximum concentration of over 100 mg L^{-1} for both labyrinthopeptins after 300 h (STY approximately 12 mg L^{-1} d^{-1} per target molecule), has very limited reproducibility (Z. Rupcic, 2017, pers. comm.) and therefore still requires further optimization. Until now, no benchmarking study on the most advantageous biotechnological cultivation conditions for the production of labyrinthopeptin has been carried out and an industrial production process for the production of labyrinthopeptins has yet to be established [42].

2.2 Submerged cultivation of filamentous bacteria

Filamentous microorganisms are of great interest to the industry because they are a rich source of valuable biotechnological products, such as antibiotics, antitumour agents, immunosuppressive agents, enzymes and organic acids. One of the most diverse bacterial groups is the division of actinobacteria, including many well-known species like *Streptomyces* and *Actinomyces*. Actinobacteria produce approximately 45 % of all the biologically active compounds isolated from microorgansisms up to now [43], showing the great importance of this bacterial group for the development of novel anti-infectives.

All Actinobacteria grow filamentously, meaning that on the micro-morphology level spores germinate and produce germ tubes, which elongate to form thread-like cells with a diameter of 0.5 to 2 µm [44] called hyphae. They grow at their tip and branch subapically [45, 46] until a more or less dense, interwoven mycelial network is developed. Depending on the inoculum and the conditions in the cultivation vessel (medium composition, pH, temperature, power input, etc.), the resulting macro-morphology in submerged culture may vary between dispersed hyphae, hyphal aggregates (so-called clumps) and dense pellets (**Fig. 2-4**). For many filamentous organisms, a clear connection between macro-morphology and productivity could be observed [31, 47–51]. However, this correlation is highly dependent on both the strain and the product. In different *Streptomyces* strains for example, dispersed and agglomerated mycelia were found to be beneficial for the production of retamycin [20], nystatin [21] and geldamycin [22], whereas the productivity of nikkomycin [23] and avermectin [24] was higher with pellets. There are also cases reported in which the productivity levels were independent from the morphology [52, 53]. An alteration of morphology during cultivation is frequently also reported [54, 55]. This is because the morphology is influenced by the environmental cultivation conditions. Those conditions are not constant throughout the cultivation and affect the organisms' physiology, which in turn affects the morphology. In batch cultivations for the production of secondary metabolites the nutrients are eventually depleted, which can change the morphological growth form. Furthermore, the increasing amount of biomass will eventually lead to mass transfer limitations, especially in dense pellets. As a result, the cells may partly not be provided with important substrates, such as oxygen, anymore. In case of non-pellet morphologies the viscosity will drastically increase, which can also become an obstacle from the process control point of view.

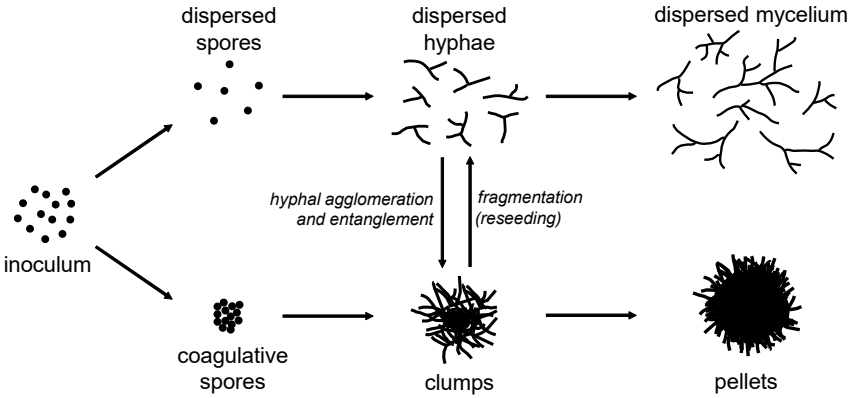

Figure 2-4: Scheme of the formation of different morphological units during submerged cultivation. An inoculum of spores can either be coagulative or non-coagulative, and germinated hyphae grow dispersed or form clumps and pellets, respectively. Clumps and pellets also develop due to hyphal agglomeration and entanglement. Reversely, shearing at the clump and pellet exterior generates hyphal fragments, also referred to as reseeding [56, 57]. Modified from [58].

2.3 Osmolality manipulation for tailored morphology

In view of the interdependence of morphology and productivity, several efforts have been made in the last decades to tailor filamentous morphology. However, the relations are complex, empirical and often not intuitive. For instance, the macro-morphology determines the micro-environment of hyphae through effects on mixing, mass transfer and broth rheology, which in turn affects product formation [59], but the mechanisms leading to formation of a certain highly productive morphology are not yet understood in detail. Several methods to tailor morphologies of filamentous microorganisms for enhanced yield of a desired product have been developed. For instance, the morphology can be influenced by the choice of nutrient sources, the input of physiological stress (e.g., by MPEC [25–28, 60]) or by other additives [29–31]. In this work it was chosen to manipulate the osmolality by the addition of inorganic salts to the cultivation broth.

The term *osmolality* refers to number of osmotically active ions/particles of solute per kilogram of solvent. The osmolality of cultivation broths is mainly dependent on the culture medium composition and changes during cultivation through consumption of nutrients, release of metabolic products and addition of an acid or base for pH control. Changes in the external osmolality trigger water fluxes along the osmotic gradient and require adaptive processes to counter either swelling or dehydration of the cells. Maintaining the hydrostatic pressure (turgor, up to 20 bar in Gram-positive bacteria [61]) is important as a positive turgor is considered as driving force for cell expansion [62]. Cells from all biological kingdoms have