Chapter 4
Bioremediation of Cucurbitacins from Cucurbitacin Phytonematicides

Phatu W. Mashela, Ebrahim Shokoohi, Ashwell R. Ndhlala, Kgabo M. Pofu, and Dineo Raphasha

Abstract Effective microorganisms (EM) had no effect on cucurbitacin content during fermentation of cucurbitacin phytonematicides. However, under field conditions, the products have short application intervals, suggesting the post application existence of bioremediation factors of cucurbitacins. The objective of the review was to investigate the factors that could be responsible for the bioremediation process of cucurbitacins from Nemarioc-AL and Nemafric-BL phytonematicides, which are novel products serving as alternatives to fumigant nematicides. The latter had been internationally withdrawn from the agrochemical markets due to their environment-unfriendliness. Among the EM constituents used during fermentation, only *Lactobacillus* species, technically, the lactic acid bacteria, were the remaining EM after the fermentation process. *Lactobacillus* species do not release reductase enzymes, which have the potential to bioremediate cucurbitacins and therefore, the existence of extended shelf life in plastic containers. Although cucurbitacin phytonematicides have long shelf lives, after field application, the efficacy is short-lived, suggesting the existence of bioremediation factors in soil environments. In the review, we noted that due to the lipophilic properties of cucurbitacins, the products could be subjected to biosorption in lipid-rich epicuticles of nematodes. Total protein of the root-knot (*Meloidogyne* species) nematode versus increasing phytonematicide concentration exhibited negative quadratic relations to the minimum point, after which the total protein increased. After biosorption to lipid-rich epicuticle by hydrophilic part of cucurbitacins, the hydrophobic part becomes predisposed to the protein-rich subepicuticular layers, resulting in isoprenylation (protein-breakdown) and after the minimum point, farnesylation (protein biosynthesis) occurred, resulting in increase of total protein. In conclusion, ecdysozoans, which are the cuticle-bearing super phylum, represented by plant nematodes in the current review, offer potential existence of bioremediation process of cucurbitacins from cucurbitacin

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phytonematicides through biosorption, isoprenylation, and farnesylation reactions, thereby opening a frontier in bioremediation of cucurbitacin phytonematicides by these microorganisms, which are numerous in the soil environment.

Keywords Biosorption · Cucurbitacins · Ecdysozoans · Farnesylation · Isoprenylation · *Lactobacillus* species · *Meloidogyne* species · Reductase enzymes · Total protein

4.1 Introduction

Cucurbitacins occur as a class of highly oxygenated triterpenoids with tetracyclic cucurbitane nucleus skeletons: (the 19-(10 → *β*)-abeo-10α-lanost-5-ene (Chen et al. 2005, 2014)), within the Cucurbitaceae Family and at least five other families (Abbas et al. 2013). Although cucurbitacins have a common nucleus skeleton, the primary cucurbitacins that are biosynthesized in plants as triterpenoids are cucurbitacin B and E, each with an acetyl function at C-25 (Gry et al. 2006). More than 20 other cucurbitacins are produced from cucurbitacin B and E through one of the following chemical reactions: hydrogenation by cucurbitacin Δ^{23} reductase, deacetylation by cucurbitacin acetylesterases, hydroxylation, dehydrogenation, and isomerization (Schabort and Teijema 1968; Dirr et al. 1986; Gry et al. 2006; Zhou et al. 2016). Generally, cucurbitacins differ from one another from hydroxylation at C-2, C-3, C-19, and C-24, the existence of a double chemical bond between C-1 and C-2 or between C-23 and C-24, the acetylation of C-25 hydroxyl groups and the presence of a ketone function at C-3 (Chen et al. 2005, 2014). The listed chemical reactions are important in explaining the potential reversal chemical processes that could enhance the bioremediation of cucurbitacins. Due to their wide-ranging biological activities, cucurbitacins have been widely investigated in pharmacological studies related to potential treatment of human diseases, particularly cancers and inflammation (Chen et al. 2005, 2014; Abbas et al. 2013; Mirr et al. 2019). In traditional medicine, cucurbitacin plant organs are widely used for treatment of various diseases (Mphahlele et al. 2012). Cucurbitacins had been recently introduced into the agriculture sector for managing plant nematode population densities as cucurbitacin phytonematicides (Mashela et al. 2017a). The latter are used as alternative to fumigant synthetic chemical nematicides, which had been internationally withdrawn from the agrochemical markets due to their degradation of ozone layer, and therefore, contributing directly to the incident of global warming (Mashela et al. 2017a).

The two cucurbitacin phytonematicides are derived from fruits of wild *Cucumis* species as crude extracts through bacterial fermentation process. The products, along with their purified active ingredients, have been widely investigated for their efficacies on suppression of plant nematodes and the amelioration of phytotoxicity

on plants using the Curve-fitting Allelochemical Response Dose (CARD) computer-based algorithm model (Liu et al. 2003; Dube and Mashela 2016; Mashela et al. 2017a). The model demonstrated that most r strategist nematode species were highly sensitive to the test products, with efficacies that have relative impact effects from 90 to 100% when compared with nematode-inoculated plants without phytonematicides. The efficacies of these products were comparable to those of synthetic chemical systemic nematicides such as aldicarb and fenamiphos (Mashela et al. 2008). In contrast, the K strategist nematode *Steinernema feltia*e, widely used as a biocontrol agent of insects and worms, is tolerant to the test phytonematicides (Madaure et al. 2018), through morphological adjustment to cucurbitacin phytonematicides (Mashela et al. 2020a). Generally, r strategist nematodes are smaller, with higher reproductive rates and shorter ontogenies, whereas the opposite is true for the K strategists (Andrews and Rouse 1982). The non-phytotoxic concentration of these phytonematicides technically referred to as the Mean Concentration Stimulation Point (MCSP), along with the application interval that successfully suppressed nematode population densities, were each nematode- and crop-specific (Mashela et al. 2017a). The MCSP values ranged from 2 to 3%, whereas application intervals ranged from 14 to 22 days (Mashela et al. 2017a). Using empirically derived application frequency values for given plant cultivars, the dosage model was developed as the product of MCSP and the application frequency, which provided the total concentration of the phytonematicide applied from the initial to the final application prior to harvest. Similarly, when using second-stage juveniles (J2) of the citrus nematode (*Tylenchulus semipenetrans*) as an indicator for application interval, the findings confirmed the empirically derived values of using MCSP and nematode ontogeny, namely, approximately two to three weeks for liquid formulation and approximately eight weeks for granular phytonematicides (Mashela et al. 2017a, b).

Granular formulations of cucurbitacin phytonematicides consistently suppressed nematode population densities when assessed at eight weeks after application (Mashela 2002; Sithole et al. 2016; Mashela et al. 2017a). However, increased population densities of *T. semipenetrans* were observed when the trial was terminated at approximately 17 weeks post application of granular phytonematicides (Maile et al. 2013). In a subsequent study using liquid formulation on *T. semipenetrans*, J2 responses to Nemarioc-AL phytonematicide and aldicarb over time exhibited negative quadratic relations. In each case, after the minima were reached, J2 densities tended to have an upswing trend, suggesting the existence of density-dependent growth patterns (Mashela et al. 2017a, b). The cited observations suggested that regardless of the formulation, cucurbitacins as active ingredients of the cucurbitacin phytonematicides could undergo degradation, which could be driven by either abiotic or biotic factors. In most cases, abiotic degradation factors for soil-drenched products like phytonematicides include soil temperature, soil type, organic matter content, and/or soil pH (Jørgensen 2007), whereas biotic factors could include microbes such as bacteria and fungi (Singh et al. 2019). The biodegradation of chemicals from the environment through living entities such as microbes is technically referred to as bioremediation (Jørgensen 2007; Odukkathil and Vasudevan 2013; Canak et al. 2019). In the current study, bioremediation of cucurbitacins from

the cucurbitacin phytonematicides was investigated to identify potential biological entities that serve as the bioremediation drivers, along with the potential bioremediation drivers.

4.2 Cucurbitacin Phytonematicides

Cucurbitacins are biosynthesized as secondary metabolites through either MEP/DOXP or Mevalonate pathway, which predominantly occur inside the mitochondria and serve as key component to ensure that the high-energy Acetyl-Co-A molecules do not accumulate at the entry site of the Krebs cycle, chemically referred to as the tricarboxylic acid cycle (TAC). Generally, a large number of secondary metabolites which are formed during glycolysis and the movement of the Acetyl-Co-A to TAC through the Mevalonate pathway or the D-Glyceraldehyde 3-phosphate through the MEP/DOXP pathway to form the triterpenoids are purposely removed as high-energy molecules from the primary metabolism pathway that takes Acetyl Co-A to TAC (Campbell and Reece 2005). The secondary metabolite pathway converts the high-energy Acetyl-Co-A or Glyceraldehyde 3-phosphate molecules into low-energy molecules such as cucurbitacins in a step-by-step conversion from precursor to precursor using specific chemical reactions facilitated by enzymatic activities in specific biosynthetic pathways (Chen et al. 2005, 2014). Since primary metabolism occurs in all living cells, precursors for cucurbitacins originate from all such cells, from where they are translocated to organs where low-energy cucurbitacins can be compartmentalized and used by resident plant organs in defense against herbivorous animals.

4.2.1 Sources of Cucurbitacin Phytonematicides

Nemarioc-AL and Nemafric-BL phytonematicides have A and B that represent active ingredients cucurbitacin A and B, respectively, with L depicting that the product is available as a liquid formulation. The two products are derived from oven-dried fruits of wild cucumber (*Cucumis myriocarpus*) and wild watermelon (*C. africanus*), indigenous to South Africa, with biodiversity center in Limpopo Province (Kristkova et al. 2003). *Cucumis myriocarpus* contains high cucurbitacin A ($C_{32}H_{46}O_9$) content in roots and fruit exclusively, with leaves being used as vegetable by the local people. In contrast, high cucurbitacin B ($C_{32}H_{48}O_8$) occurs in all organs of *Cucumis africanus*.

4.2.2 Preparation of Cucurbitacin Phytonematicides

Harvested fruits from the two plant species are cut into pieces and preserved through oven-drying. In medicinal plants, the materials are dried at 40 °C for 72 h in order to preserve the active ingredients (Müller and Heindl 2006). However, when fresh fruit pieces from the two *Cucumis* species were dried at 40 °C, the hyphae of the fungus that is resident on fruits of *Cucumis* species, namely, *Penicillium simplicissimum,* proliferated, resulting in rotting of the material (Mphahlele et al. 2012). The suitable temperature where *P. simplicissimum* would not thrive, which also resulted in the optimization of cucurbitacins, was derived at 52 °C for 72 h (Shadung et al. 2016). After drying, raw materials were ground separately in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to pass through a 1 mm sieve and stored in closed containers (Shadung and Mashela 2016). Fruits of the two plant species were separately fermented using effective microorganisms (EM) that comprised yeast bacteria, lactic acid bacteria, photosynthetic bacteria, and actinomycete bacteria, with each playing a distinct role in ensuring that the final product was of high quality. Raw materials used in the fermentation process of the two phytonematicides were summarized to enhance clarity on the inputs of the products (Table 4.1). The 20 L air-sealed plastic container had an outlet dangled in a 2 L bottle half-filled with water to allow for the escape of toxic gaseous end-products produced during the fermentation process.

4.2.3 Role of EM Components During the Fermentation Process

Fermentation refers to metabolic processes whereby organic molecules are converted into acids, gases, or alcohol in the absence of electron transport chain that requires oxygen. In simple terms, fermentation pathways regenerate the coenzyme nicotinamide adenine dinucleotide (NAD^+), which is used in cytoplasmic glycolysis to release energy in the form of adenosine triphosphate (ATP). Generally, fermentation during glycolysis yields a net of two ATP molecules, while mitochondrial

Table 4.1 Raw materials for preparation of cucurbitacin phytonematicide in a 20 L plastic container

Material	Nemarioc-AL	Nemafric-BL
Ground *Cucumis* fruits	80 g	40 g
Chlorine-free water	16 L	16 L
Sugar	100 g	100 g
Molasses	100 mL	100 mL
Effective microorganisms	100 mL	100 mL

respiration in the presence of oxygen yields the grand total of 38 ATP molecules from one glucose molecule. The organic molecules in phytonematicides include sugars in the form of purified sugars and molasses, cellulose, lignin, starch, and various other secondary metabolites, which EM constituents should biodegrade and release into solution during the fermentation process in chemical processes which are driven by various enzymes, but which are out of the scope of the current work.

Lactic acid bacteria hydrolyze the toughest materials in cell walls of plants, namely, cellulose and lignin (Madigan and Martino 2006). The final product of cellulose and lignin fermentation is lactic acid, which promotes the reduction of pH of the phytonematicide solution (Higa and Parr 1994). Yeast bacteria also reduce pH by hydrolyzing glucose to pyruvic acid through the anaerobic glycolysis pathway (Stetter 2006). The latter, without oxygen cannot proceed to Krebs cycle, the tricarboxylic acid cycle (TAC) that serves as the final electron acceptor during the enzyme-driven release of energy in the form of adenosine triphosphate (ATP) molecules, for use in biological activities. The pH of the finished liquid phytonematicides gradually decreased from 7.0 to 3.7 in 14 days after fermentation. Such conditions favor the thriving of bacteria at the expense of fungal growth (Madigan and Martino 2006). Yeast bacteria also release antimicrobial chemicals that add to the sterilization of the finished phytonematicide by preventing fungal growth that might even include the elimination of the previously described *P. simplicissimum* in cucurbitacin phytonematicides. During fermentation, the materials release copious sulfur as hydrogen sulfur (H_2S), where NAD of photosynthetic bacteria is reduced to NADH by H^+ ion from H_2S during Photosystem II (Campbell and Reece 2005). The released S is oxidized to form SO_4^{2-}, which is a lethal gas (Stetter 2006). Soon after its formation, SO_4^{2-} is reduced by $2H^+$ from H_2O molecule to form a strong acid, sulfuric acid (H_2SO_4), with highly corrosive capabilities. The Gram-positive actinomycete bacteria, which are oxygen-tolerant, have the capability to release chitinase that hydrolyzes chitin in exoskeletons of insects, insect eggs, nematode eggs, and mycelia of various fungi (Madigan and Martino 2006). Apparently, the constituents of EM have no biodegradation capabilities on cucurbitacins, for otherwise EM would not be suitable for use.

4.2.4 Unique Features of Cucurbitacin Phytonematicides

Cucurbitacin A is partially polar and slightly soluble in water, whereas cucurbitacin B is non-polar and insoluble in water (Chen et al. 2005). Both cucurbitacin A and B have high boiling points at sea level (760 mmHg = one atmosphere sea level), which occur at 731 °C and 699 °C, respectively (Krieger 2001). In contrast, at sea level, methyl bromide and Nemacur boil at 3.56 °C and 49 °C, respectively (Pesticidal Manual 1979; Windholz 1983). Generally, when *Cucumis* fruit pieces are oven-dried at 52 °C, the precursors and their related enzymes continued down their respective biosynthetic pathway, with subsequent formation of the low-energy cucurbitacins as demonstrated during the storage of raw materials of the

cucurbitacin phytonematicides in sealed and unsealed plastic containers (Shadung and Mashela 2016). Similarly, containerized phytonematicides during the shelf life studies had limited degradation rate, except under chilled conditions (Mashela et al. 2020b).

4.2.5 Shelf Life of Cucurbitacin Phytonematicides

Cucurbitacin phytonematicides did not conform to the Arrhenius model, established for shelf life of various products, with product quality versus time exhibiting negative linear relations (Labuza and Riboh 1982; Steel 2004). Under chilled conditions (5 °C, 95–98% RH), Nemarioc-AL phytonematicide degraded rapidly with negligent shelf life (Mashela et al. 2020b). In most cases, cucurbitacin A breaks down rapidly into cucumin ($C_{36}H_{46}O_9$) and leptodermin ($C_{36}H_{46}O_8$) (Jeffrey 1980). However, Nemafric-BL phytonematicide with its stable cucurbitacin B versus storage time exhibited positive quadratic relations with shelf life spans of 35 weeks under chilled conditions (Mashela et al. 2020b). Under fixed tropical conditions (38 °C, 90% RH), Nemarioc-AL and Nemafric-BL phytonematicides versus time exhibited positive quadratic relations with shelf life spans of approximately 35 and 825 weeks, respectively. Extended shelf life in the two test phytonematicides were temperature-dependent, with tropical conditions being the most favorable for the storage of the products, which is well suited for use in tropical regions, where plant nematodes abound. The observed shelf life spans suggested the existence of unique features in both products when stored under tropical conditions. In contrast, with daily sampling for cucurbitacin during a 15-day period, cucurbitacin E-glycoside versus time exhibited negative quadratic trends, which were also temperature-depended (Martin et al. 2002).

4.3 Bioremediation Drivers of Cucurbitacin Phytonematicides

Bioremediation of pesticides has been defined as a process where the active ingredients are removed from the environment by microorganisms through biodegradation or biosorption processes, thereby decontaminating the environment (Ying 2018). Such processes reduce or eliminate the efficacy of the products against the target pests (Ying 2018). Historically, bioremediation factors include plants, bacteria, and fungi (Davison 2005; Kvesitadze et al. 2006; Juwarkar et al. 2010; Odukkathil and Vasudevan 2013; Singh et al. 2019), driven by factors such as microbe type, temperature, nutrition, enzymes, antimicrobial chemicals, types of chemical reaction such as the redox reactions and the size of the chemical compounds (Norris 1993; Varjani and Upsani 2017). In some cases, bacteria that release

acids during bioremediation have the tendency to eliminate pathogenic fungi but could also eliminate other essential bacteria through the production of antimicrobial chemicals as by-products (Slonczewki et al. 2009; Chen et al. 2020). The focus of the current study, bioremediation factors of cucurbitacins, focused on both conventional and unconventional factors. The latter involved running short-term experiments to validate claims, which are being advanced in the study.

4.3.1 Potential Effects of Plants on Bioremediation of Cucurbitacins

Bioremediation of chemicals using plants is referred to as phytoremediation, which occurs in one of three forms, namely, phytoextraction, phytotransformation, and rhizodegradation (Vidali 2001; Kvesitadze et al. 2006). In phytoextraction, the test chemical accumulates in organs of the plant, referred to as phytoaccumulation (Kvesitadze et al. 2006). In contrast, phytotransformation and rhizodegradation each results in degradation of the chemicals without absorbing them and are technically referred to as phytodegradation (Kvesitadze et al. 2006). In living plants, cucurbitacin A was stored in fruit and roots of *C. myriocarpus,* whereas cucurbitacin B is stored in all organs of *C. africanus* plants (Jeffrey 1980). In chemical residue studies of the cucurbitacin phytonematicide in tomato production, cucurbitacin A and B were hardly detected in tomatoes or foliage of indigenous vegetable, nightshade (*Solanum nigrum*) (Dube and Mashela 2016; Shadung et al. 2017). In olives and strawberries, chemical residues of another non-polar triterpenoid, azadirachtin ($C_{35}H_{44}O_{16}$), were also not detected (Caboni et al. 2002, 2006). Generally, non-polar molecules, including glucose, cannot be transported through the bipolar membranes in the symplastic pathway of the endodermis into or out of the vascular bundle (Campbell and Reece 2005). In a tomato-cowpea or tomato-sweet stem sorghum rotation, where nematode population densities on tomato plants were managed using the cucurbitacin phytonematicides, chemical residues of cucurbitacins stimulated growth of cowpea plants (Mashela 2014) and sweet stem sorghum as successor crops (Mashela and Dube 2014). Observations in the cited two last studies suggested that phytoextraction and rhizodegradation of cucurbitacins in plants hardly occurred. Generally, the rate of bioremediation is influenced by numerous abiotic and biotic factors, including the size of the chemical compound (Varjani and Upsani 2017). Due to the large size of cucurbitacins and other triterpenoids, these chemicals probably have slower rates of biodegradation.

The two phytonematicides, when applied using empirically based concentration and application interval, did not suppress nematode population densities indefinitely, thereby necessitating the need for the establishment of the application intervals (Mashela et al. 2017a). The latter had been empirically designed in such a manner that the products would interrupt the ontogeny of the test nematode at least once, depending on the length of ontogeny of the managed nematode species. Generally, the application interval of the test phytonematicides is approximately

14–19 days for *Meloidogyne* species, which suggest the existence of cucurbitacin degradation processes in the soil as induced by either abiotic or biotic factors, or both. Plants infected by *Meloidogyne* species usually release copious concentration of amino acids into the rhizosphere, thereby modifying the rhizosphere through reducing soil pH (Wallace 1973). Acidic conditions in the rhizosphere of such plants might suppress most fungal pathogens while promoting bacterial growth, some of which might play undocumented roles in bioremediation processes of cucurbitacins.

4.3.2 Bioremediation of Cucurbitacins by Effective Microbes

At 35 weeks after storage of the phytonematicides, components of the constituents of EM from Nemafric-BL phytonematicide solution were subjected to the phylogenetic tree constructed based on maximum-likelihood analysis of 16S rRNA gene (Shokoohi 2020, unpublished data). Briefly, the South African strain of *Lactobacillus* was clustered with other *Lactobacillus* species that included *L. vini* and *L. mobilis,* along with unidentified *Lactobacillus* species (Fig. 4.1). Comparison of the 16S rRNA gene sequence of *Lactobacillus* isolates from Nemafric-BL phytonematicides corresponded with *L. mobilis* (acc. nr. AB242320) from the GenBank database at 87% similarity with 151 nucleotide differences. Besides, with *L. vini* (acc. nr. AY681132) the test strain had 87% similarity with 151 nucleotide differences. The 16S rRNA nucleotide sequence BLAST had a similarity from 85 to 87% with molecular strains of *Lactobacillus* species in the Genbank, without trace of most other bacteria that were used in the fermentation process of Nemafric-BL phytonematicide. In EM, to protect the intellectual property, the constituents of EM were provided in general terms, without providing the species names (Higa and Parr 1994). In the current study, *Lactobacillus* species, commonly called lactic acid bacteria, displayed all other components of EM, suggesting the existence of a consortium of *Lactobacillus* species in the constituents of EM. In mutant bitter Hwakesbury watermelon (*Citrullus lanatus* Thumb. Naakai (syn. *Citrulus vulgaris* Schad) study, *Bacillus* species remained as the dominant bacteria without affecting cucurbitacin E-glycoside content in the extracted solution (Martin et al. 2002).

Most bioremediation processes involve oxidation-reduction (redox) reactions, characterized by the existence of electron donors and electron acceptors (Vidali 2001). In order to enhance the understanding of why *Lactobacillus* species eliminated the other EM constituents, let us briefly review how the species achieve this feat. Fermentation is the process that includes two phases, first is the breakdown of glucose (glycolysis) to pyruvate molecules, with the net gain of two ATP and two NADH molecules in the cytoplasm of bacteria. The ATP molecules are used by *Lactobacillus* species for biological activities such as movement, feeding, and reproduction. In the second phase of fermentation, the produced NADH donates its electron in the form of H to the pyruvate molecules to convert them to lactic acids,

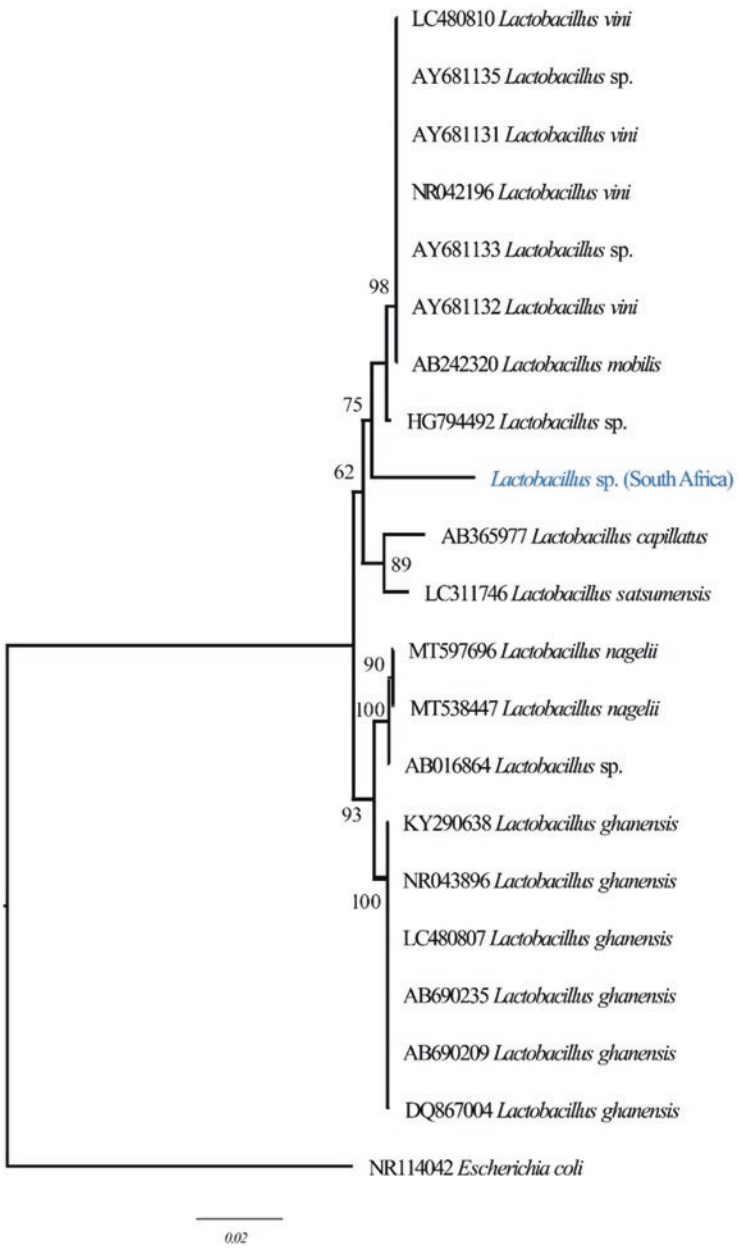

Fig. 4.1 Phylogenetic tree of South African strains of *Lactobacillus* species (blue color) derived from Nemafric-BL phytonematicide using Neighbor Joining method through MegaX software (Shokoohi 2021, unpublished data)

with NAD^{2+} being regenerated for re-use during glycolysis as an electron acceptor to allow the production of ATP to continue in the cytoplasm (Fig. 4.2).

In *Lactobacillus* species, the fermentation process had been described as being either homolactic or heterolactic fermentative process, where glucose molecules are metabolized through the phosphoketolase pathway as explained below (Vidali 2001):

$$\text{Homolactic fermentative process}: \text{glucose} + 2\,\text{ADP} + \text{Pi} \rightarrow 2\,\text{lactic acids} + 2\,\text{ATP}$$

$$\text{Heterolactic fermentative process}: \text{glucose} + \text{ADP} + \text{Pi} \rightarrow \text{lactic acids} + \text{ethanol} + CO_2 + \text{ATP}$$

Energy-wise, the homolactic fermentation process is the more efficient than the other is, since one molecule of glucose is metabolized to two molecules of lactic acids and two molecules of ATP as end-products with Pi being the phosphorus derived from the substrate. In contrast, during the heterolactic fermentation process, one glucose molecule is metabolized to one lactic acid, one CO_2, and one ATP as end-products (Vidali 2001). In addition to the formation of the released acids and hydrogen peroxide (H_2O_2), which also suppresses anaerobic bacteria except *Lactobacillus* species that are oxygen-tolerant. The latter also produce copious quantities of bacteriocins, salivaricins, and sodium butyrate, which inhibit growth of pathogenic microbes, including bacteria, fungi (Barbour et al. 2020), providing an explanation why the cucurbitacin phytonematicide is highly aseptic cucurbitacins known to be biodegraded by reductase enzymes (Ellis, 2002), but such enzymes are hardly produced by *Lactobacillus* species (Ellis 2002). Just to emphasize, in addition to other reductase-producing microorganisms (Yum et al. 1999), which are not part of the EM constituents, there could be many other bioremediation factors in the soil, which would help in the explanation of the loss of efficacy in the test products over time (Mashela et al. 2017a).

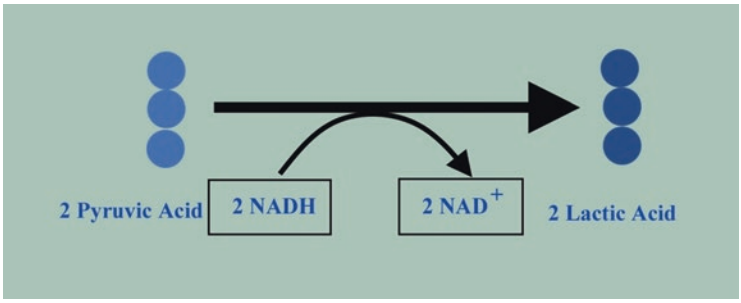

Fig. 4.2 Lactic acid fermentation uses pyruvic acid and NADH to generate NAD^+ and lactic acids, with NAD+ being reused in glycolysis to produce NADH and ATP, where ATP is used for biological activities—all in the absence of oxygen. The circles represent C atoms

4.3.3 *Bioremediation of Cucurbitacins by Ecdysozoans*

Ecdysozoans constitute one of the major groups within the Kingdom Animalia and include eight phyla, with the commonly reported being the Arthropoda, Kinorhyncha, Loricifera, Priapula, Onychophora, Nematomorpha, Nematoda, and Tardigrada (Ruggiero et al. 2015). Unlike other animals that build rigid skeletons using mineral elements, ecdysozoans build the cuticles as exoskeletons using organic material. Such exoskeletons are thinner and lighter than mineral skeletons, and therefore, do not require joints to allow flexibility as is the case in mineral skeletons. However, exoskeletons are sufficiently rigid to prevent growth of the body and therefore, ecdysozoans regularly shed off their cuticles, a process called ecdysis (molting), controlled by steroid hormones called ecdysteroids (Niwa and Niwa 2014). In the current work, we used the nematode cuticle to expound how ecdysozoans could play a role in bioremediation of cucurbitacins from the test cucurbitacin phytonematicides.

4.3.3.1 Nematode Cuticles

The nematode cuticle consists of four main layers, the outer layer (epicuticle), cortical layer, the collagen layer (with 4 distinct sublayers), and the hypodermal layer (Perry and Moens 2013). Only the epicuticle and median layers are shed off during the molting process (Fig. 4.3), whereas the hypodermis is used to generate the new cuticle (Schultz et al. 2014). The newly molted juvenile exits the shed cuticular layer through the stoma. The epicuticle is formed by lipids, which are coated with a glycoprotein, technically referred to as a surface coat that plays a protective role to the epicuticle. Lipids in the epicuticle enhance mobility of nematodes in aqueous solutions due to their incompatibility (Schultz et al. 2014). The collagen has four protein-rich sublayers, (a) cortical layer with insoluble proteins called cuticulins, (b) median layer with pillar-like proteins filled with gelatinous matrix, (c) basal layer with distinct soluble proteins in the form of fiber and those as dense gelatinous matrix (Fig. 4.3).

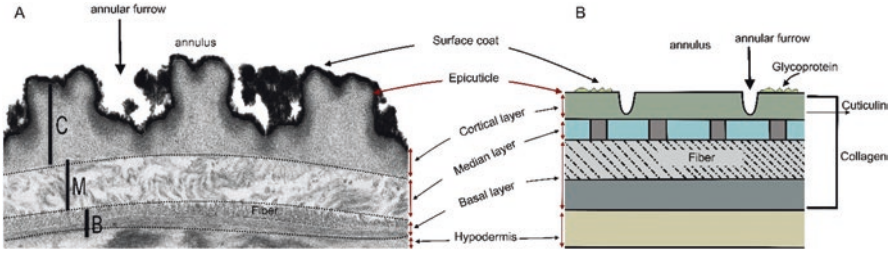

Fig. 4.3 Schematic nematode cuticle being molted (**a**) and cuticle layers (**b**) of *Caenorhabditis elegans* from transmission electron microscopy photograph (Schultz et al. 2014, improved from Shokoohi 2019)

4.3.3.2 Role of Epicuticle in Bioremediation of Cucurbitacins

Cucurbitacins are lipophilic (Van Wyk and Wink 2004), which confers them the status of being hydrophobic (Patel et al. 2009). The two properties improve the loading capabilities of cucurbitacin drugs (Patel et al. 2009). Lipids in the epicuticles have the capability of attracting cucurbitacin molecules from aqueous solutions and therefore their chemical status of being lipophilic. This attraction provides sufficient explanation why nematodes are highly sensitive to cucurbitacin phytonematicides in crude and purified forms (Dube and Mashela 2016, 2017, 2018; Dube et al. 2019). The attraction of cucurbitacins by the epicuticles removes the active ingredients of cucurbitacins from the environment, which agrees with the description of bioremediation processes (Jørgensen 2007; Canak et al. 2019). Once cucurbitacins are removed from the environment by the epicuticles, they can, due to their hydrophobic properties, further be attracted to proteins in the middle layers, which are replete with proteins, and therefore, well-suited for these roles, referred to as isoprenylation and farnesylation reactions.

4.3.3.3 Role of Subcuticular Layers in Bioremediation of Cucurbitacins

Isoprenylation is the addition of hydrophobic molecules such as cucurbitacins through the prenyl groups (3-methylbut-2-en-1-yl) to the proteins (Marshall 1993; Casey and Seabra 1996; Novelli and D'Apice 2012), whereas the addition of lipids to proteins is lipidation. The prenyl groups are important for protein-to-protein binding through specialized prenyl-binding domains. Prenylation involves the transfer of either a farnesyl or a geranylgeranyl moiety to C-terminal cysteine(s) of the target protein, using one of three enzymes, namely, farnesyl transferase, Caax protease, and geranylgeranyl transferase I (Novelli and D'Apice 2012). Importantly, farnesyl is one of the enzymes required in the biosynthesis of cucurbitacins through the MEP/DORXP pathway (Chen et al. 2005).

4.3.3.4 Evidence of Isoprenylation and Farnesylation in Nematodes

After exposing J2 of the southern root-knot nematode, *M. incognita*, to a geometric series (0, 2, 4, 8, 18, 32 and 64%) of Nemafric-BL phytonematicides for 72 h, total protein was determined using TruSpecCHNS Macro (Leo, St. Joseph, MI, USA) (Mashela and Shokoohi 2021). Briefly, data were subjected to analysis of variance using SAS software to establish the significance at 5% level probability. Prior to subjecting the data to lines of the best fit, data expressed as exponentials (2^0, 2^1, 2^2, 2^3, 2^4, 2^5 and 2) were transformed using $\log_{2^x} = x$ (1) to homogenize the variance (Causton 1977). Using $x = -b_1/2b_2$ relation from the quadratic relation, $Y = b_2x^2 + b_1x + c$, the minimum total protein was accrued at 4.9% (transformed data) phytonematicides. During isoprenylation, which breaks down the proteins, there was a gradual decrease of total proteins (Fig. 4.4). After the minimum, the

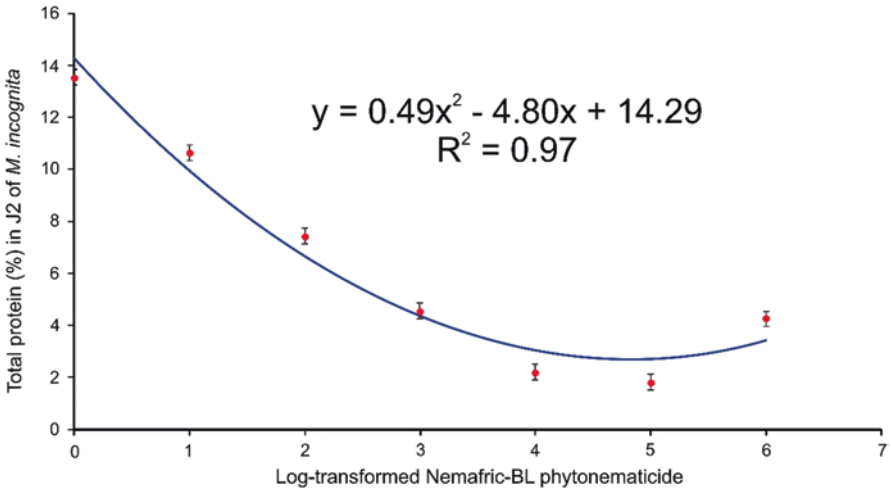

Fig. 4.4 Response of total protein to increasing concentration of Nemafric-BL phytonematicide (Mashela and Shokoohi 2021)

total protein started to increase, thereby supporting the view of farnesylation. The latter is a type of isoprenylation where a post-translational modification of proteins in which an isoprenyl group is added to a cysteine residue, which is an important process to mediate protein-to-protein interactions, thereby increasing total proteins to enable protein-to-protein membrane interactions to occur (Marshall 1993; Casey and Seabra 1996; Novelli and D'Apice 2012). Both isoprenylation and farnesylation as observed in this study supported the principles of density-dependent growth patterns, which occur when biological entities are subjected to increasing concentration of allelochemicals such as cucurbitacins (Liu et al. 2003; Mashela et al. 2017a).

4.4 Conclusion and Future Perspectives

Bioremediation processes involving secondary metabolites of phytonematicides involve various processes. Such processes require some knowledge of the biosynthetic pathways, including the precursors and enzymes involved in such processes, along with enzymes that can play a role in the reversal of the processes. *Lactobacillus* species do not produce reductase enzymes that have capabilities of hydrolyzing cucurbitacins, but the soil is replete with reductase-producing microorganisms, which could play some role in bioremediation process of cucurbitacins. In the current study, supported by empirical-evidence, we concluded that in addition to other potential bioremediation factors of cucurbitacins, the ecdysozoans play an active role in bioremediation of cucurbitacins through biosorption, isoprenylation, and farnesylation reactions due to the unique properties of cucurbitacins and cuticles.

Future studies with other cuticle-bearing organisms in the super phylum ecdysozoans would provide an essential explanation on limited persistence of cucurbitacin phytonematicides when applied under field conditions.

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