STUDY ON

WILDLIFE PRODUCTS PRODUCED FROM SYNTHETIC OR CULTURED DNA

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Background

1. This study was commissioned by the CITES Secretariat in implementing Decision 17.89-17.91 on *Specimens produced from synthetic or cultured DNA*, and to respond to the comment made by the CITES Animals and Plants Committees[1] that the definitions of synthetic or cultured DNA were not self-evident and would need to be clarified.

2. This study follows the detailed terms of reference that was finalized by the Standing Committee after its 69th meeting in late 2017, with the exception that the ordering of the sections was altered to address the scientific issues first, and the legal/policy considerations afterwards[2].

3. The study uses terms that are commonly used by molecular biologists, but are not defined in CITES; furthermore, some terms are difficult to define using the vocabulary of the Convention. While effort has been made to bring the language of this document closer to that used in CITES, some terms have been left deliberately vague in order to serve the purpose of the study. For example, the definition of the term "organism" used in this document is derived from the definition for "living organism" defined in the Cartagena Protocol on Biosafety[3], which means "any biological entity capable of transferring or replicating genetic material, including sterile organisms, viruses and viroids". This could denote a whole population of a given taxon or an individual animal/plant, depending on the context which has been used.

4. It is, or will soon become, possible to synthesize tissue from any organism, including animals and plants covered by CITES, using the techniques of modern biotechnology. This may include modifying the DNA in the tissue and/or using cell culture technology. In some circumstances, the culture of whole cells will be sufficient to provide a product that is identical (or essentially equivalent) to that found in the living organism – an example could be a powder which is derived from rhinoceros horn. In others, the tissue requires

[1] At the joint session of the 29th meeting of the Animals Committee and the 23rd meeting of the Plants Committee (Geneva, July 2017)
[2] For details on the terms of reference and the background to this study, see AC30 Doc.14/PC24 Doc.14.
[3] http://bch.cbd.int/protocol/

some structure, which will require multiple variants of cells derived from the original organisms and the use of a scaffold or the use of a 3D printing technique to grow the cells in an appropriate three-dimensional structure. The technologies available to sequence and modify the genomes of organisms also provide an opportunity to replicate organisms that are endangered.

> *"Is the new technology to be welcomed because it holds the possibility of novel and radical solutions to global problems such as the perfect storm of shortages in food, water and energy resources or is it to be feared, for the impact of novel organisms and associated new economic arrangements on ecosystems and rural societies?"*[4]

5. The technologies allow biological systems to be used in commerce and industry, that *could* have a significant impact on conservation.

    a. It is possible to clone individual organisms and therefore to maintain endangered populations in containment.

    b. The 'poaching', illegal or unsustainable use or destruction of organisms to feed an insatiable quest for their tissues (or derived products) could (arguably – see later) be assuaged through synthetic production of these tissues or products

6. On the other hand, these technologies may be used for *laundering* of listed products where synthesised versions hide illegitimate usage. In addition, the presence on the market of manufactured artefacts could increase the market for the wild species. Caution is also needed as the introduction of engineered products could have an unwanted impact on the ecosystem in which the wild organism occurs.

Part 1. Description of different ways that DNA can be synthesized, cultured or otherwise produced artificially, and how wildlife products can be produced from synthetic or cultured DNA in the context of CITES

Introduction

7. This section attempts to look at the science which is used to derive products from synthetic tissues – which may include modification of the DNA that is found in all tissues. It embraces a vast field, and therefore provides footnotes pointing to many original articles to provide context.

8. There are real differences between the manner in which those working at the whole organism and ecosystems level and those practising modern biotechnology[5], including

[4] Adapted from Redford KH, Adams W, Carlson R, Mace GM and Ceccarelli B (2014) "Synthetic Biology and the conservation of Biodiversity" Fauna & Flora International, Oryx **(48)3** 330-336

[5] The Cartagena Protocol on Biosafety to the Convention on Biological Diversity defines "Modern biotechnology" as the application of:

a. In vitro nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or

b. Fusion of cells beyond the taxonomic family,

synthetic biology, approach their disciplines. "Many of those working in synthetic biology may not have detailed knowledge of biological structure, function, diversity or management at ecosystem or even organism level."[6] An understanding of what can now be attempted in producing variants of organisms, growing cells and tissues and modifying these for use by society is driving synthetic biology in a manner (possibly) very different from those intent on looking at our world from an ecological viewpoint and cognisant of our biological heritage and understanding the total ecological spectrum in which an organism exists, even where the sustainable use of biological diversity is an aim.

9. Precaution and attitudes to risk often differentiate those who are involved in the cutting edge of molecular and synthetic biology and those in conservation. "This culture of caution is critical to conservation's future engagement with synthetic biology and it underpins specific debates about the use or release of organisms"[6]. Precaution requires that where there are threats of serious or irreversible damage to the environment, "lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation"[7] Scientists working in a laboratory environment may not need to address this issue, but those using the properties of species that interact with their environment have precaution as a starting position where their exploitation may impact adversely of the ecosystem in which they are found.

10. The **genetic material (DNA)** in cells is primarily that which determines inheritance from generation to generation. The majority of this genetic material is found in the nucleus of eukaryotic cells with a small but important amount in the mitochondria and chloroplasts. The sequence of the DNA is highly conserved from generation to generation, and cells contain systems to both conserve and repair DNA.

11. **Cell culture** is the process by which cells are grown under controlled conditions, generally outside their natural environment. Cultures of plant cells can, and are used, to generate whole plants. Cultures of animal and human cells are now significant tools**:** Different variants of cell culture are used in modelling disease, IVF technology, stem cell and cancer research, monoclonal antibody production, regenerative medicine and therapeutic protein production[8].

12. **Tissue culture** is different, as it may involve a number of different cell types which form the tissue. The early part of twentieth century was the time when the basic principles for plant and animal cell cultures *in vitro* were developed. An understanding both of the techniques to be used to obtain stable cell cultures and of the changes in the cells during multiple cell-cycles was achieved in the latter half of the twentieth century. Tissue culture or the culture of organs is different from cell culture which in essence provides either a layer of cells on gel or suspension of the cells in liquid culture. Tissues require some form

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that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection;"

[6] Radford K (2014) "Synthetic biology and the conservation of biodiversity" Fauna & Flora International Oryx July 2014, **48(3)** 330-336

[7] the Rio Declaration on environment and development (1992) Principle 15r

[8] Jedrzejczak-Silicka M (2017) "History of Cell Culture" in "New Insights into Cell Culture Technology" Downloaded from: http://www.intechopen.com/books/new-insights-into-cell-culture- technology Chapter 1 http://dx.doi.org/10.5772/66905

of supporting structure in order to develop the characteristic structures in three dimensions.

Background on the science

13. During the life-cycle of an individual higher organism changes occur within the DNA molecules. These include epigenetic changes which alter the expression of genes, and changes to the ends of chromosomes (telomeres) which limit the number of cell divisions that can occur (termed senescence)[9].

    a. Non-genetic factors contribute to many cellular functions, traits and phenotypes[10]. "Epigenetic[11] change is a regular and natural occurrence but can also be influenced by several factors including age, the environment/lifestyle, and disease state. Epigenetic modifications can manifest as commonly as the manner in which cells differentiate to end up as a variety of cell types, from specialist brain cells to skin cells, liver cells, etc. Epigenetic change can have more damaging effects that can result in diseases like cancer. At least three systems including DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing are currently considered to initiate and sustain epigenetic change"[12]

    b. "Telomeres and their associated proteins serve to camouflage the chromosome ends from the DNA repair machinery that would otherwise identify them as double strand breaks, and from exonucleases that might recognize them as substrates."[13] They therefore protect the chromosome, but there is an attrition of telomere length with each cell division which results in cell death after a number of divisions. (i) telomeres shorten at each cell division due to incomplete replication of their ends; (ii) they are shortened by oxidative damage; and (iii) when telomeres. reach a critical length, cells enter a senescent state and cell division ceases. [14] This implies that normal mammalian cells (in particular) can only undergo a finite number of cell divisions before ceasing to divide. There are also likely to be changes in the cells during replication.

14. The term *'bioengineering'* has been used by many to refer to the deliberate modification of the DNA in organisms, and in this document is used as a generic term for the many different ways to modify organisms. In some countries genetic modification has been

[9] Telomeres are most commonly composed of non-coding tandemly repeated sequences. For humans, the telomeric sequence is TTAGGG, which extends in a 5' to 3' direction from the double-stranded DNA to a single stranded region.

[10] Stricke SH , Köferle A and Beck S, From profiles to function in epigenomics, NATURE Reviews Genetics **18** (January 2017) 51- 66

[11] Epigenetics is the study of heritable changes in gene expression (active versus inactive genes) that do not involve changes to the underlying DNA sequence

[12] https://www.whatisepigenetics.com/fundamentals/

[13] M Shawi, C Autexier, (2008) "Telomerase, senescence and ageing" Mechanisms of Ageing and Development **129** 3–10

[14] M J.P. Simons (2015) "Questioning causal involvement of telomeres in aging", Ageing Research Reviews 24 (2015) 191–196

variously described as genetic engineering or genetic manipulation. Gene editing, described later as a significant advance in modifying genetic material would also fall within this definition.

Technology (i): DNA Modification

15. During the last few decades it has become possible to modify the DNA of both eukaryotic and prokaryotic organisms using a variety of techniques. The modification of the DNA of bacterial cells (prokaryotic organisms) has been extensively studied and has become commonplace in the laboratory and in commerce.

16. Modification of the DNA of dicotyledonous **plants** was, until recently primarily achieved through the use of a bacterium, *agrobacterium tumifaciens,* that inserts a DNA sequence into that of a plant using a piece of circular DNA (Ti plasmid).[15] The position of insertion is imprecise. The use of a marker that enables modified cells to be distinguished from those either unaffected or modified in a deleterious fashion (because of the insertion in an inappropriate position in the DNA of the plant) is important as the plant can often be regenerated from a single cell. Further sexual crossing of the modified plant with unmodified relatives ensures that any unwanted modifications can be identified and excluded before commercialisation (see a publication of the Australian Office of gene technology regulator[16])

17. DNA modification of animals has always been challenging. The technology was initiated in the 1980s; a detailed examination of genetically modified animals is addressed in a publication of the Netherlands Commission on Genetic Modification (COGEM):[17]

> *"The most common route for producing a GM genetically modified (GM) animal is to inject foreign DNA into a fertilised egg, also known as 'microinjection'. For mammals, injected eggs are placed into a 'foster' mother where they develop to term and offspring are born normally, carrying the extra, foreign DNA. This DNA is now part of a chromosome, so when the GM animal mates and produces offspring, the transgene is inherited in the same way as any other DNA and a line of GM animals is bred that carries the extra DNA. The first GM animal, a mouse, was made in the early 1980s, and this technology has been successfully applied to most mammals, including cattle, pigs and sheep, poultry, fish, and also Drosophila and other insects."*

[15] Gelvin B. S. (2003) "Agrobacterium-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool". Microbiology and Molecular Biology Reviews 67(1): 16–37 -

A. tumefaciens Ti plasmids have been produced that lack the genes responsible for gall formation (disarmed plasmids). Genes to be inserted into the plant are put into the T-DNA section of these disarmed plasmids. A. tumefaciens cells carrying such plasmids cannot produce a gall in an infected plant but will transfer the T-DNA sequence carrying the genes of interest into the plant cell where they stably integrate into the plant genome

[16] http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/plant-modifications-ref-1-htm

[17] Genetically Modified Animals: A Wanted and Unwanted Reality. COGEM topic report (2012) CGM/120111-01

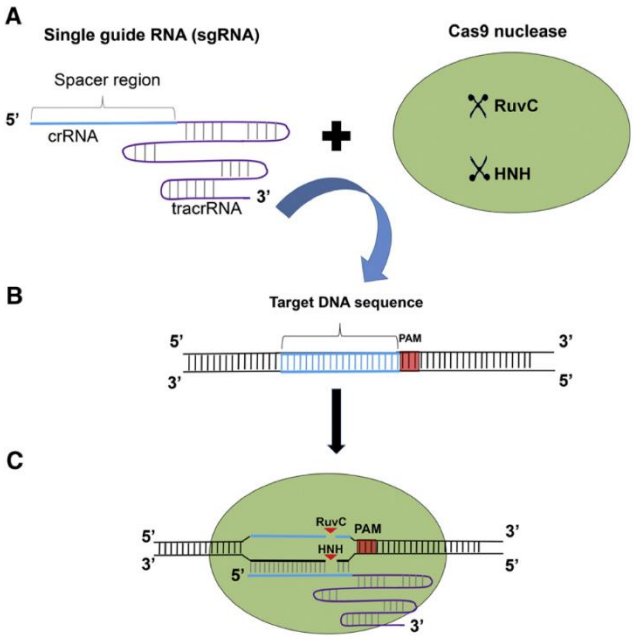
18. The ability to edit genomic DNA inside cells has been limited by the dearth of effective tools which break the chains of DNA at specific sites[18]. However, this has changed during the last few years, resulting in a revolution due to the ability to determine the sequence of the DNA precisely and easily, to manufacture DNA sequences on demand, and modify DNA in organisms in a *precise* manner. "Since 1975, reading and writing platforms have exhibited increases in throughput of three-million-fold and one-billion-fold" and a million-fold reduction in cost in the past 10 years in both reading and writing"[19].

19. The major advance in the editing of DNA to modify the DNA expression is that of **CRISPR/Cas9[20]** technologies. The advent of targetable nucleases (enzymes that can break the DNA chains) has given researchers the ability to induce specific double-strand breaks in the DNA.[21] It is now possible to "directly edit or modulate the function of DNA sequences in their endogenous context in virtually any organism of choice, enabling them to elucidate the functional organization of the genome at the systems level, as well as identify causal genetic variations." [22] Cas9 is one of a group of RNA-guided endonucleases. A short strand of RNA complementary to the double stranded DNA directs the cleavage of the phosphodiester bond within both strands of the polynucleotide chain. As the DNA targets are recognized via RNA-DNA base pairing, changing the sequence of the guide RNA easily alters DNA specificity and therefore, specific sites within the genome can be targeted.[23] "Researchers create a small piece of RNA with a short guide sequence that attaches (binds) to a specific target sequence of DNA in a genome. The RNA also binds to the Cas9 enzyme. As in bacteria, the modified RNA is used to recognize the DNA sequence, and the Cas9 enzyme cuts the DNA at the targeted location. Although Cas9 is the enzyme that is used most often, other enzymes (for example Cpf1) can also be used. Once the DNA is cut, researchers use the cell's own DNA repair machinery to add or delete pieces of genetic material, or to make changes to the DNA by replacing an existing segment with a customized DNA sequence."[24]. [25] The discovery of the CRISPR system has led to the modification of the Cas9 protein and the discovery of similar molecules that increase the precision of the cut in the double stranded DNA significantly.

20. It is the specificity of this technology that is important if it is used for conservation, cloning, therapy in animals and humans, industrial manufacturing or even for food. Much

[18] Carroll, D. (2014). Genome engineering with targetable nucleases. Annual Reviews. Biochem. 83, 409–439.
[19] Chari, R., and Church, G.M. (2017). Beyond editing to writing large genomes. Nature Reviews Genetics volume 18, pages 749–760
[20] CRISPR is an abbreviation of Clustered Regularly Interspaced Short Palindromic Repeats. CRISPR – Cas9 is an abbreviation for CRISPR-associated protein 9.
[21] Carroll D (2013) "Staying on target with CRISPR-Cas" Nature Biotechnology **31**(9) 807-809
[22] Hsu P, Lander ES, and Zhang F (2014) Development and Applications of CRISPR-Cas9 for Genome Engineering , Cell 157, June 5, 1262
[23] Sternberg SH and Doudna JA (2015) "Expanding the Biologist's Toolkit with CRISPR-Cas9" Molecular Cell **58**, 568-574. http://dx.doi.org/10.1016/j.molcel.2015.02.032
[24] https://ghr.nlm.nih.gov/primer/genomicresearch/genomeediting
[25] A detailed article on the modern methods of editing DNA, beyond the scope of this report can be found in Gupta RM, Musunuru K (2014). Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. The Journal of Clinical Investigation. 2014;124(10):4154-4161. doi:10.1172/JCI72992.

work has been going on to improve efficiency of the process and ensure that modifications to the DNA are as specific as possible and at the desired site in the DNA. [26]



Schematic representation of the CRISPR/Cas9 complex. (A) The CRISPR/Cas9 system requires a single guide RNA (sgRNA) and CRISPR-associated protein 9 (Cas9) containing two nuclease domains, RuvC and HNH. sgRNA consists of CRISPR RNA (crRNA) (indicated by the blue bold line) and a trans-activating crRNA (tracrRNA) (indicated by the purple bold line). The crRNA region is also called the spacer region. (B) The spacer region in the crRNA is complementary to a 20 bp-long target DNA sequence, which is known as the protospacer (indicated by the blue bold line). The target DNA sequence is immediately followed by a short sequence known as the protospacer adjacent motif (PAM) (indicated by the red box). (C) sgRNA targets the complementary target DNA sequence, and Cas9 nuclease follows to generate a double stranded break at the target site.[27]

21. The technologies are thought to allow replacement of most earlier methods for modifying the DNA (or RNA) in organisms, . It is possible to introduce genes into specific sites in the genome which alter the characteristics of an organism.

    a. This could be used to 'resurrect' organisms through hybridisation with related organisms.

[26] Carroll, D (2013). "Staying on target with CRISPR-Cas: four independent studies shed light on the specificity of RNA-guided genome editing tools based on the Streptococcus pyogenes Cas9 protein." Nature Biotechnology, vol. 31, no. 9, 2013, p. 807 Academic OneFile, http://link.galegroup.com/apps/doc/A344207979/AONE?u=unict&sid=AONE&xid=64ddcd3c. Accessed 24 Mar. 2018.,

[27] Young-Il, Bharathi Suresh, Hyongbum Kim, Suresh Ramakrishna (2015) "CRISPR/Cas9 system as an innovative genetic engineering tool: Enhancements in sequence specificity and delivery methods" Biochimica et Biophysica Acta 1856 (2015) 234–243

b. Organisms that are close to extinction could be modified by the addition of a variety of genes or by their deletion to adapt them to changed environments and in theory, increase fecundity

c. Genes can be extracted from organisms that need conservation and added to less threatened related organisms so that pressure on the former is relieved through production of specific products without the need to harvest regulated organisms

22. **Gene drive** is an expansion of these technologies that allows for a trait to be propagated throughout a population. "Gene drives are systems of biased inheritance in which the ability of a genetic element to pass from a parent to its offspring through sexual reproduction is enhanced. Thus, the result of a gene drive is the preferential increase of a specific genotype, the genetic makeup of an organism that determines a specific phenotype (trait), from one generation to the next, and potentially throughout the population.[28] – Indeed, a wide variety of gene drives occur in nature that can cause genetic elements to spread throughout populations to varying degrees. Researchers are studying how to harness such natural mechanisms (e.g., transposable elements, homing endonucleases, and meiotic drive) to develop gene-drive modified organisms. Preliminary evidence suggests that gene drives developed in the laboratory with CRISPR/Cas9 could spread a targeted gene through nearly 100% of a given population of yeast, fruit flies, or mosquitoes.[28]

23. DNA is not only altered through mutations in the sequence. Changes to the DNA or it associated structures can occur, termed epigenetic changes. "Epigenetics is the study of a group of mechanisms that affects how genes are 'read' by cells. It's the term used to explain how a gene expresses an organism's characteristics (active versus inactive genes) and to what degree. Epigenetics is akin to "directing"—it orchestrates how genes work, which shapes the behaviour of all organisms. It also describes heritable changes in gene expression that do not involve changes to the underlying DNA—a change in phenotype without a change in genotype—which in turn affects how cells read the genes. At least four systems—methylation, non-coding RNA (ncRNA)-associated gene silencing, histone modifications and chromatin remodelling of DNA—are currently considered to initiate and sustain epigenetic change."[29] It is likely that an understanding of epigenetics will play a role in the survival or endangered species in the laboratory, and ultimately in the wild.

24. Whilst the set of technologies already described are used for altering the genetic material (possibly modifying the germline and hence becoming heritable), **synthetic biology** involves many different technologies. "As well as molecular biology, synthetic biology interfaces with engineering, chemistry, physics, computer science and systems biology and is focused on developing more rapid and simple methods to produce genetically modified organisms (GMOs) by adding or removing genes, or creating genetic elements

[28] National Academies of Sciences, Engineering, and Medicine. 2016. Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values. Washington, DC: The National Academies Press. doi: 10.17226/23405.

[29] https://geneticliteracyproject.org/category/epigenetics/?mc_cid=615b71adba&mc_eid=cc680d1eb4 (accessed 6 May 2018)

from scratch"[30] Amongst the many definitions of synthetic biology, in 2016 the Royal Society defined synthetic biology as "The design and construction of novel artificial biological pathways, organisms and devices or the redesign of existing natural biological systems"[31] The European Group on Ethics in Science and new technologies defined it as "A new research field within which scientists and engineers seek to modify existing organisms by designing and synthesising artificial genes or proteins, metabolic or developmental pathways and complete biological systems in order to understand the basic molecular mechanisms of biological organisms and to perform new and useful functions".[30] These new developments could have positive or negative impacts on the long-term survival of the species in the wild.

Technology (ii): Cell culture

25. It is not necessarily only the modification of DNA that could drive the production of desired tissues. It may be advances in the way cells can be isolated, grown and exploited that is already being used for multiple purposes

26. The selection, isolation and culturing of particular cell types is crucial where tissues from organisms are to be grown; in some instances, cells may be isolated and grown in culture and assembly of a tissue might be induced. In other cases, assembly of a specific tissue may involve numerous steps. The first step would be to isolate specific cell types. Tissues involve multiple different cells, and the *creation* of a tissue may require culturing of multiple cell types in isolation from one another and their combination using some form of scaffolding to simulate the tissue.

27. There has been a long history of culturing the cells of living organisms. A culture is a method of multiplying living cells by letting them multiplicate in predetermined culture media under controlled laboratory conditions. Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favourable artificial environment. A culture may originate from a single cell, in which case all of the cells are likely to be the same as one another (clones). If the starting material is heterogenous, many different cell types (or even organisms) may be present in the cultured medium.

28. There are many different cells in a multicellular organism. For example, it is estimated that there are 37.2 trillion cells in the human body. Attempts at identifying these indicate that there are at least 300 variants amongst these myriads in the human body[32]

29. For a unicellular organism, the culturing and growth of the organisms over many 'generations' has been studied for over a century, and for many of these organisms, the conditions required to allow replication in the laboratory have been described and are well understood. The **bacteria** that can be grown in the laboratory are only a small

[30] Science for Environment Policy (2016) Synthetic biology and biodiversity. Future Brief 15. Produced for the European Commission DG Environment by the Science Communication Unit, UWE, Bristol. Available at: http://ec.europa.eu/science-environment-policy

[31] Science for Environment Policy (2016) Synthetic biology and biodiversity. Future Brief 15. Produced for the European Commission DG Environment by the Science Communication Unit, UWE, Bristol. Available at: http://ec.europa.eu/science-environment-policy, page 7

[32] S Connor (2017) in "The Cell Atlas" MIT Technology Review, **120(2)** p59-61

fraction of the total diversity of organisms that exists in nature[33] There are many unculturable organisms where the conditions for growing them in the laboratory have not, as yet, been identified – "the culturing efforts of the last 2 centuries had managed to replicate permissive growth conditions for only a small subset of the total bacterial diversity."[29] An understanding of the techniques necessary for growing unicellular organisms, including those considered *unculturable* could provide the information necessary for conservation in the future.

30. For many **plants**, it is possible to regenerate a complete plant from a single cell. "Many somatic plant cells, including some fully differentiated types (e.g. leaf mesophyll), provided they contain intact nuclear, plastid and mitochondrial genomes, have the capacity to regenerate into whole plants. This phenomenon is totipotency, an amazing developmental plasticity that sets plant cells apart from most of their animal counterparts and was first demonstrated by Steward and Reinert in the 1950s."[34] Most plants at most stages of the life cycle have some populations of cells that are totipotent.

31. In order for plant cells to grow in culture, many of the chemicals normally available within the plant must be made available to the cells in the medium. The selection of chemicals is made so as to promote the growth of particular cells. Pieces of plant tissue will slowly divide and grow into a colourless mass of cells if they are kept in special conditions. While the regeneration of a whole plant from a single cell is possible, the process requires specific plant hormones to stimulate **totipotency**.[35] Loss of totipotency in plant cells is probably due to genetic changes, including physical changes to chromosomes, for example loss of DNA, nucleotide substitution, endopolyploidy) or epigenetic changes, which are changes in gene expression as a consequence of development, for example DNA methylation).[34]

32. The stable introduction of new genetic material into breeding programs for **animals** is now possible through some form of germ line modification. Genetic modification of embryonic stem cells in culture followed by the production of chimeric animals using blastocyst injection is a tool for this modification.[36] Nuclear transfer enabled the production of clones (Dolly the sheep) and could be used to increase the number of animals within an endangered species. The addition of DNA material can be achieved through the injection of desired genes into the pronucleus of a zygote. It does not always work, as integration may not occur during the first cell cycle resulting in mosaic embryos. The position of insertion in the genome is essentially random, which can both disrupt other genetic processes or make the expression of the desired trait variable. The advent of Crispr/Cas9 technologies is expected to hugely increase the scope of possible modifications of particular cells within an organism.

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[33] EJ Stewart (2012) "Growing unculturable bacteria" J Bacteriol. 2012 Aug;194(16):4151-60. doi: 10.1128/JB.00345-12. Epub 2012 Jun 1.

[34] http://plantsinaction.science.uq.edu.au/edition1/?q=content/10-2-1-concept-totipotency

[35] totipotency (Lat. *totipotentia,* "ability for all [things]") is the ability of a single cell to divide and produce all of the differentiated cells in an organism. Spores and zygotes are examples of totipotent cells. In the spectrum of cell potency, totipotency represents the cell with the greatest differentiation potential. (https://en.wikipedia.org/wiki/Cell_potency)

[36] Campbell KHS, Wilmut I, (1996) "Totipotency or multipotentiality of cultured cells: Applications and progress" Theriogenology **47**, Issue 1, 1 January 1997, Pages 63-72

33. It is possible to isolate animal cells and culture them. Normal animal cells usually divide only a limited number of times (approximately 30 times) before losing their ability to proliferate, which is a genetically determined event known as senescence. Almost 60 years ago Hayflick was able to show that cultured human cells have limited capacity to divide – the limitation in number of cell divisions has become known as the Hayflick limit. Some cell lines become immortal through a process called transformation, which can occur spontaneously or can be chemically or virally induced. When a finite cell line undergoes transformation, and acquires the ability to divide indefinitely, it becomes a continuous cell line. Cell immortalization is a very complicated cellular process and the exact biological mechanisms are still largely not well understood.[37]

34. If attempts are made to culture a primary cell population past the Hayflick limit, the cells would have to be modified in some way in order to circumvent senescence[38]

    a. Cancer cells are spontaneous immortalised cells that have undergone genetic changes that make them *immortal.* Hence the expression of an immortalising oncogene in the normal cell could enable a continuous cell line

    b. Many viral genes affect the cell cycle and can be used to avert senescence through repression of some enzymes involved in the cell cycle. The most effective way of inducing immortality in cell lines is through viral infection.

    c. Express an immortalizing oncogene in normal cells.

    d. Shortening of telomeres (in mammals, thousands of repeats of TTAGGG) would appear to lead to senescence. The most common immortality gene is telomerase which is able to extend the telomeres and hence (probably) enable the cells in culture to undergo infinite cell divisions. "Normal human cells stably expressing transfected telomerase can maintain the length of their telomeres, and exceed their maximum lifespan by more than 5-fold."[39]

<u>Example application of cell culture (rhino horn and elephant ivory)[40]</u>

35. The horns of most animals have a bony core covered by a thin sheath of keratin, the same substance as hair and nails. Rhino horns are unique, however, because they are composed entirely of keratin, with areas of melanin and calcium deposits that help maintain the structure.

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[37] Applied Biological Material Inc, "General Guidelines for Cell Immortalization" see http:''www.abmgood.com

[38] McLean J.S. (1999) Immortalization Strategies for Mammalian Cells. In: Jenkins N. (eds) Animal Cell Biotechnology. Methods in Biotechnology™, vol 8. Humana Pressi

[39] Shay JW and Wright WE (2000) "Hayflick his limit and cellular ageing" Nature Reviews – Molecular Cell Biology , **1,** 72-76

[40] Sources:
Ryder ML (§962) "Structure of Rhinoceros Horn" Nature volume 193, 1199–1201
Hieronymus TL, Witmer, LM and Ridgely, Ryan C. (2006) "Structure of white rhinoceros (Ceratotherium simum) horn investigated by X-ray computed tomography and histology with implications for growth and external form" Journal of Morphology, October 2006, 267(10), 1172-1176
Raubenheimer E, Bosman M, Vorster R and Noffke C (1998) "Histogenesis of the chequered pattern of ivory of the African elephant (Loxodonta Africana)" Archives of Oral Biology 43 (1998) 969-977

36. The rhinoceros horn is not, however, simply a clump of modified hair; it most closely resembles the structure of horses' hoofs, turtle beaks and cockatoo bills. If the cells forming this horn are isolated, immortalised and grown in culture, arguably a powder could easily be derived that might be used as authentic rhino horn. An elephant tusk, on the other hand, resembles a tooth, enormously enlarged maxillary incisors, the growth of the tusk is continuous throughout life and its size at any age is dependent on the sex of the animal, the rate of attrition and breakage of the tooth, as well as genetic and environmental factors.

37. The implication of the above example is that simple cell culture to produce either rhino horn or the basics of elephant tusk could not provide anything resembling the products sought after by collectors of ivory or of rhino horn unless the desired product is a powder derived from a specific tissue.

Conversion of somatic cells into stem cells

38. Stem cells are undifferentiated cells that have the potential to develop into many different cell types in the organism during early life and growth. In addition, in many tissues they serve as a sort of internal repair system, dividing essentially without limit to replenish other cells. Only fertilised embryos and blastomeres up to the 8-cell stage are considered to be totipotent cells in humans[41]

39. When a stem cell divides, each new cell has the potential either to remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell.[42] These are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. They can be induced to become tissue- or organ-specific cells with special functions. Embryonic stem cells were first identified in 1981; by 1998 a method had been devised to isolate stem cells from human embryos and culture them in the laboratory. "In 2006 researchers made another breakthrough by identifying conditions that would allow some specialized adult cells to be "reprogrammed" genetically to assume a stem cell-like state. These are termed induced pluripotent stem cells (iPSCs)." Embryonic stem cells are pluripotent. Adult stem cells are thought to be limited to differentiating into the different cell types of their tissue of origin.[43]

40. If somatic cells, found in normal tissue, could be converted into stem cells, it would be possible to grow any organ, or even arguably, be used as the basis for embryos which could regenerate the whole animal. For example, Takahashi *et al* argue that "Successful reprogramming of differentiated human somatic cells into a pluripotent state would

[41] Jong Soo Kim, Hyun Woo Choi, Sol Choi, Jeong Tae Do (2011) "Reprogrammed Pluripotent Stem Cells from Somatic Cells" International Journal of Stem Cells **4** ,1-8.

[42] https://stemcells.nih.gov/info/basics/1.htm

[43] NIH Stem Cell Information Home Page. In *Stem Cell Information* [World Wide Web site]. Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services, 2016 [cited March 27, 2018] Available at < //stemcells.nih.gov/info/basics/5.htm>

allow creation of patient- and disease-specific stem cells."[44] They were able to show that "Human iPS cells were similar to human embryonic stem (ES) cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. Furthermore, these cells could differentiate into cell types of the three germ layers in vitro and in teratomas. These findings demonstrate that iPS cells can be generated from adult human fibroblasts."

41. The ability to isolate pluripotent stem cells, even where modification of the cells has been used to induce pluripotency is an important step in the regeneration of organs or tissues which may then be used in industrial or commercial contexts

Technology (iii): Tissue (Organ) culture

42. Since its emergence in the mid-1980s, tissue engineering has exploded as an exciting and multidisciplinary field aiming to develop biological substitutes to structure, and even restore, replace or regenerate defective tissues. [46]

43. Tissues and organs contain a multiplicity of different cell types. There is interaction between these cells which is important for their function. Tissues and organs are also three dimensional. The absence of the normal tissue microenvironment can alter cellular responses. Three-dimensional cultures that position cells on synthetic matrices, or organoid or organ-on-a-chip cultures, in which different cell spontaneously organize contacts with other cells and natural matrix only partly overcome this limitation.[45] The media used for culture are the same as for cell and tissue culture, yet trying to keep organs or parts of organs in culture requires the provision of nutrients and oxygen, possibly deep within the tissue.[46] Stimulation of cells in culture to produce 'structures' similar to those produced *in vivo'* remains difficult.

44. In order to maintain the relationships between cells in culture they need to be grown in some kind of three-dimensional matrix. In embryonic tissue there is self-assembly, where the cells grow in such a way as to form an organ – involving self-assembly. This is not easily achieved starting from cells in culture. Scaffolds represent important components for tissue engineering. "Apart from blood cells, most, if not all other, normal cells in human tissues are anchorage-dependent residing in a solid matrix called extracellular matrix (ECM). There are numerous types of ECM in human tissues, which usually have multiple components and tissue-specific composition."[46] The different cells that form a tissue are necessarily put together in a ratio and spatial arrangement similar to that found in the original tissue. There are many problems with organ culture, including

[44] Takahashi et al (2007) "Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors" Cell 131, 861–872, November 30, 2007 861-872

[45] Al-Lamki RS, Bradley JR and Pober JS (2017) "Human Organ Culture: Updating the Approach to Bridge the Gap from In Vitro to In Vivo in Inflammation, Cancer, and Stem Cell Biology." Front. Med. **4**:148. doi: 10.3389/fmed.2017.00148

[46] Chan BP and Leong KW (2008) "Scaffolding in tissue engineering: general approaches and tissue-specific considerations" Eur Spine J **17** (Suppl 4):S467–S479 DOI 10.1007/s00586-008-0745-3

a. Organ cultures, in general, cannot be propagated, hence for each 'experiment' there is a need for a fresh organ from a donor.
b. Variations are high, and reproducibility is low.
c. Difficult to prepare, besides being expensive

45. Any artificial scaffold needs to fill the tissue void, to provide structural support and to deliver growth factors and/or cells that have the ability to form tissues[47] The table below indicates that which is observed in vivo, and that which is required of an extracellular matrix on which the cells can proliferate.

The extra-cellular matrix in tissue *in vivo*	**Analogous functions of scaffolds in engineered tissues**
Provides structural support for cells to reside	Provides structural support for exogenously applied cells to attach, grow, migrate and differentiate *in vitro* and *in vivo*
Contributes to the mechanical properties of tissues	Provides the shape and mechanical stability to the tissue defect and gives the rigidity and stiffness to the engineered tissues
Provides bioactive cues for cells to respond to their microenvironment	Interacts with cells actively to facilitate activities such as proliferation and differentiation
Acts as the reservoirs of growth factors and potentiates their actions	Serves as delivery vehicle and reservoir for exogenously applied growth-stimulating factors
Provides a flexible physical environment to allow remodelling in response to tissue dynamic processes such as wound healing	Provides a void volume for vascularization and new tissue formation during remodelling[46]

46. It is possible to derive artificial organs which mimic the layout of organs found in living systems using three-dimensional printing techniques where the organ is built up layer by layer using ink-jet printing technologies. It allows for using different cell types to be placed in anatomically correct positions relative to others (equivalent to different colour inks).

<u>Example of organ printing technologies</u>[48,49]

47. The custom designed Integrated Tissue and Organ Printing (ITOP) system consists of a sophisticated nozzle system with a resolution of 2-50 µm that deposits cell-laden hydrogels together with biodegradable polymers. The cell matrix - consisting of gelatine, fibrinogen, hyaluronic acid and glycerol – is optimized to promote cell survival and differentiation whilst providing support and uniform dispersion. Mechanical shape and strength is provided by poly(r -caprolactone) polymers, which were chosen for their low

[47] Howard D, Buttery LD, Shakesheff KM and Roberts SJ (2008) "Tissue engineering: strategies, stem cells and scaffolds" J. Anat. **213** 66–72 doi: 10.1111/j.1469-7580.2008.00878.x

[48] Nature Reviews Rheumatology I Published online 3 Mar 2016; corrected on line 9 March 2016; doi:10.1038/n rrheum.2016.29. - Kao,g, H.W. et al.(2016) A 3D bioprinting system to produce human scale tissue constructs with structural integrity. Nat. Biotech. http;//dx.doi.ocg/10.1038/nbL3413

[49] Graham AD *et al* (2017) "High-Resolution Patterned Cellular Constructs by Droplet-Based 3D Printing" *Scientific Reports* **7**, Article number: 7004(2017) doi:10.1038/s41598-017-06358-x

melting temperature. After printing, the addition of a thrombin solution induces crosslinking of the fibrinogen and the unlinked components are then washed away.

Example application of tissue culture (beef)[50]

48. "Made with some breadcrumbs, egg, and 20,000 lab-grown cow muscle cells, the world's first lab-grown burger made its debut last year. It was a proof of concept, evidence that you can make meat in lab. The technology is too difficult and expensive to show up at grocery stores any time soon. In the future, however, proponents hope so-called cultured meat will get cheaper. If it does, making beef from stem cells could be an environmentally friendly alternative to, you know, killing animals for food." [1] "Producing cultured meat for processed meat products, such as sausages, burgers and nuggets should be comparatively simple, whereas cultured meat which should be more highly structured, such as for an *in-vitro* steak is considerably more of a challenge".

Example application of tissue culture (rhino horn)[51]

49. There is much hype about producing rhino horn in order to produce rhino horn equivalents that may impact on the disastrous poaching and killing of rhinoceros in the wild. "There's a startup called Pembient that is 3D printing rhinoceros horns in a lab on the far edge of San Francisco. These are not horns that look like rhino horns. These are genetically identical rhino horns, according to the startup", and , from Africa geographic: "A Seattle-based biotechnology startup that hopes to grow cruelty-free rhino horns in a laboratory to save the animal from poachers — and eventually from extinction — says it has already produced multiple batches of rhino horn powder." The article also indicates that "If all goes well, the company wants to grow elephant ivory and even tiger bones". Rhinoceros Horn LLC proposes, "We've teamed up with the world's leading developer of keratin products, Keraplast Technologies, and using Replicine™ Functional Keratin® have produced a keratin protein powder that is biologically identical to the keratin from rhino horn. But unlike real rhino horn, Replicine™ Functional Keratin® is sustainably produced with no harm to animals and is proven in peer review medical publications to provide health benefits."

Example application of tissue culture (elephant ivory)[52]

50. Another group of scientists have been examining the cellular and molecular structure of ivory in order to examine the possibility of creating synthetic ivory in the laboratory which would, to all intents and purposes mimic or even be indistinguishable from the real thing. "To move from a biological to an engineered material, it is critical to understand the relationships between structure and function," says Buehler, who has worked on spider silk but has no connection to the ivory researchers. "It is often hard to actually manufacture a material that mimics the one found in nature." The key technical challenge for understanding a natural material like ivory, he adds, is to have access to a range of

[50] Sources:
Diep F (2014) "What Does It Take To Make Meat From Stem Cells?" Popular Science May 21, 2014.
http://www.futurefood.org/in-vitro-meat/index_en.php
https://www.futuremeat.org/resources

[51] Sources:
Buhr S (2015) Biotech Startup Pembient Is Making Rhino Horns, Sans Rhino"
https://techcrunch.com/2015/04/27/cuzscience/
https://africageographic.com/blog/biologist-aims-to-grow-synthetic-rhino-horns/
https://www.savetherhino.org/latest_news/news/1508_synthetic_rhino_horn_will_it_save_the_rhino

[52] Sources:
Smithsonian Institute "Appalled by the Illegal Trade in Elephant Ivory, a Biologist Decided to Make His Own" https://www.smithsonianmag.com/science-nature/faking-elephant-ivory-180963226/

imaging techniques that allow the material to be characterized from the molecular to the macro levels."

Part 2. Relevant scientific/technological elements to be considered in the context of CITES

51. Where products are manufactured synthetically or derived from CITES-listed species, Regulation will depend on the nature of the product in comparison to that derived from the listed species as to whether they:

    a. are indistinguishable. These would primarily be chemicals, whether high value or simple. This remains important as the synthetic manufacture may relieve pressure on natural organisms. There would be no way in which the synthetic product could be distinguished from that derived from the wild.

    b. could have some sort of ''kite-mark' or biological barcode inserted so that they can easily be distinguished, preferably by simple tests so that border controls can be effective. This could be, for example, slight changes to protein sequence that do not occur naturally and do not have functional impact but are readily observed e.g. colour change

    c. have inherent characteristics that enable differentiation e.g. epigenetic differences that are easily detected or sequences in the DNA for which probes can be made which easily distinguish the synthetic from the natural. Once again, the insertion of a 'kite-mark' would make differentiation relatively simple.

    d. the regularity of the cell structure may allow the distinction between synthetic and wild-based products could be used as a marker

52. Where it is impossible to differentiate between products derived from CITES regulated species and synthetic products, the synthetic product should be considered as regulated as the original product. Where they can be differentiated, some form of regulation may still be necessary.

53. There are many uses of the new technologies described in the above section that may have an impact on the implementation of CITES, directly or otherwise. On one hand, they could be used to replace products that are currently derived from species regulated by CITES, which may result in reducing pressure on the species in the wild, while continuing to fulfil the market with the desired product. On the other hand, the commercial availability of the synthetic product may lead to an increased demand of the product, driving some suppliers to mix wild-sourced products into the market.

54. Tissues or artefacts that are manufactured synthetically are likely to impact on the 'original' product – whether this would increase the likelihood of greater or lesser exploitation of the endangered species depends on the ability to distinguish the two, so that different regulatory measures can be applied. This is possible in most, but not all cases.

<u>Chemicals</u>

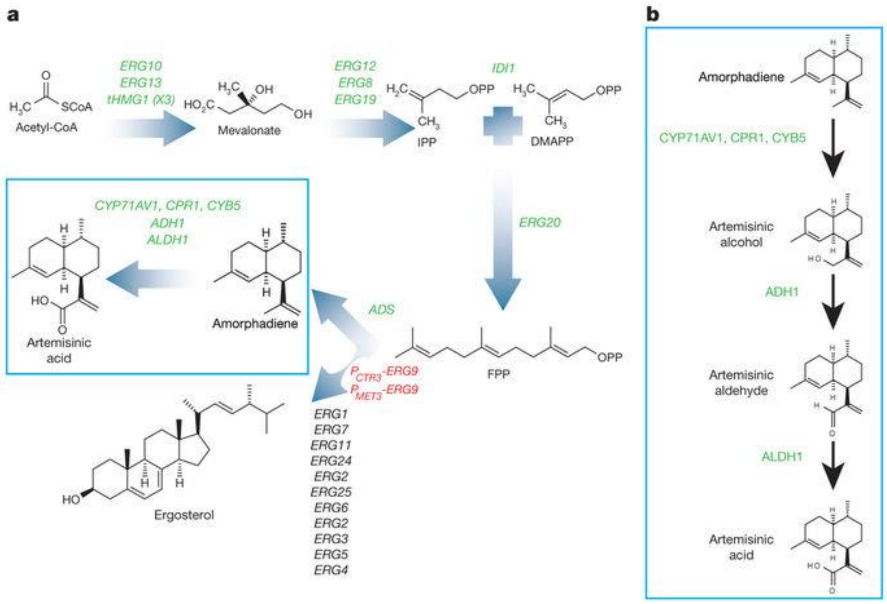
55. Technologies could be used to produce chemicals extracted from CITES-listed specimens. They are purified ingredients/compounds that could take on the form of e.g., oils, acids,

salts, alkaloids, antibiotics, and so on. They may be used in different sectors, including cosmetics, pharmaceuticals, fragrance, and food processing.

56. As the chemicals are purified, markers cannot be used to indicate the origin of the product. In these instances, there may be no difference between the product produced artificially and that derived from the organism. The chemicals could be synthesised in the laboratory or produced using cell culture of tissues derived from the original organism or from microbes engineered to produce the desired compound. The chemicals could themselves then be modified ensuring greater selectivity for a specific purpose:

    a. "Microorganisms have become an increasingly important platform for the production of drugs, chemicals, and biofuels from renewable resources. Advances in protein engineering, metabolic engineering, and synthetic biology enable redesigning microbial cellular networks and fine-tuning physiological capabilities, thus generating industrially viable strains for the production of natural and unnatural value-added compounds." These could include "synthesis of valued-added products including alkaloids, terpenoids, flavonoids, polyketides, non-ribosomal peptides, biofuels, and chemicals." [53]

    b. An example would be the synthetic production of arteminisin produced by the plant *Artemisia annua*[54] The authors "determined the full artemisinic acid biosynthetic pathway and developed a process for the production of the antimalarial drug artemisinin by fermentation of simple inexpensive carbon substrates using engineered *S. cerevisiae* to produce artemisinic acid, followed by extraction and chemical conversion to artemisinin.[54]



[53] Jing Du and Zengyi Shao (2011) "Engineering microbial factories for synthesis of value-added products" J Ind Microbiol Biotechnol. 2011 Aug; 38(8): 873–890

[54] Paddon C J *et al* (2013) "High-level semi-synthetic production of the potent antimalarial artemisinin" Nature **496**, 528–532 (25 April 2013) doi:10.1038/nature12051

c. A second example of high value chemicals that are now produced synthetically are musks. "Synthetic musks, known as white musks in the perfume industry, are a class of synthetic aroma compounds to emulate the scent of deer musk and other animal musks (ambergris, castoreumand civet). Synthetic musks have a clean, smooth and sweet scent lacking the faecal notes of animal musks. They are used as flavourings and fixatives in cosmetics, detergents, perfumes and foods, supplying the base note of many perfume formulas. Most musk fragrance used in perfumery today is synthetic" [55]

d. Technologies could be used to produce herbicides or pesticides that impact as minimally as possible on non-target organisms through increased selectivity and better understanding of their mode of action. For example, the information derived from the DNA of the CITES protected species could arguably be used to design chemicals used in agriculture which specifically have little or no effect on particular species.

e. Microbial cells can be altered to produce specific chemicals – a simple example is the production of vitamin C (ascorbic acid) using yeasts[56] There is a huge diversity in microbial cell types that could be used as 'cell factories' - bacteria, archae, yeast and filamentous fungi. These 'factories' are used in structural biology, food microbiology, natural products, biomining, nanotechnology and biosensing, for example. [57] Although microbial cells are not subject to the requirements of the Convention, their products could either alleviate the stress on endangered organisms through their no longer being required or even increase the commercial stress on the *real* products

f. "Plant sources of most high-value natural products (NPs) are not domesticated and therefore their production cannot be undertaken on an agricultural scale. Further, these plant species are often slow growing, their populations limiting, the concentration of the target molecule highly variable and routinely present at extremely low concentrations". Techniques for culturing the cells of such plants and for modifying, either through the techniques described in the previous sections or through identifying the conditions that enable efficient production of the desired products are now well-understood. Modifying the expression of genes in such plants could permit their commercial exploitation, ensuring the survival of the original plants in the wild. The identification of plants that are endangered, but which produce NPs, and for which exploitation could lead to extinction is important so that the techniques described in this paper could be used to alleviate the environmental pressure.

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[55] https://en.wikipedia.org/wiki/Synthetic_musk (accessed 6 May 2018)

[56] Sauer M *et al (2004)* "Production of l-Ascorbic Acid by Metabolically Engineered *Saccharomyces cerevisiae and Zygosaccharomyces bailii"* Appl Environ Microbiol. 2004 Oct; 70(10): 6086–6091. doi: 10.1128/AEM.70.10.6086-6091.2004

[57] Maurilio De Felice *et al* (2008) "The scientific impact of microbial cell factories" Microb Cell Fact. 2008; 7: 33. Published online 2008 Dec 1. doi: 10.1186/1475-2859-7-33

g. Impurities in the chemical extracts of natural-sourced products may permit differentiation from synthetic products, which may only contain the active (target) chemical compound.

Proteins

57. Proteins could be synthesised and be available commercially:

a. The proteins could be indistinguishable from those derived from nature but in some instances it may be desirable, in order to protect the native species, to require minor changes to be made to the protein sequence of the synthetically produced protein such that the modification does not impact in a negative manner on its efficacy (whatever the purpose of production) but could be used for detection purposes.

b. The production of proteins within organisms is likely to be influenced by the environment, and **gene drive** could be utilised in order to minimise the impact of altered environments (such as that induced by climate change). Caution would have to be exercised, as the modification could result in invasion of non-indigenous habitats. An example of an analysis taking this into account is an examination of the impact of climate change on the proteins and gene expression of some freshwater fish.[58]

Cells, tissues, and higher (up to whole organisms)

58. The technologies enable the production of specific cells or tissues, modified or otherwise, which could be used to produce products that impact on CITES protected species. As identified in a previous section, the technologies could be used to immortalise these cells, allowing the culture and production of vast quantities of desired cells. Genetic markers could be inserted into the genome of the cultured products which could allow for simple tests as to whether a product has been synthesised or derived from the endangered organism. For some complex multi-cellular products, the absence of contaminants and the regularity of the cell structure may allow the distinction between synthetic and wild-based products. 3D printing technology could be used to mimic irregularities and contaminants in the synthetic product.

59. It would theoretically be possible to replicate specific tissues, such as skin, wood, ornamental plants, fur, corals for jewellery and even meat:

a. The culturing of ornamental plants such as orchids (which require a particular micro-environment) has advanced significantly in recent years.[59]

[58] Jesus TF *et al* (2017) "Protein analysis and gene expression indicate differential vulnerability of Iberian fish species under a climate change scenario" PLoS One. 2017 Jul 18;12(7):e0181325. doi: 10.1371/journal.pone.0181325. eCollection 2017.

[59] For a review, see *Recent Advances in Orchid Tissue Culture*: Semiarti, Endang & Purwantoro, Aziz & Indrianto, Ari. (2015). IN VITRO CULTURE OF ORCHIDS: THE ROLES OF CLASS-1 KNOX GENE IN SHOOT DEVELOPMENT A REVIEW. Journal of Biological Researches. 20. Or Recent Advances in Orchid Tissue Culture https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=5&ved=0ahUKEwi-

b. Reptile skin is covered with scutes or scales which, along with many other characteristics, distinguish reptiles from animals of other classes. Scales are made of alpha and beta keratin and are formed from the epidermis (contrary to fish, in which the scales are formed from the dermis) [60].

c. In order to produce muscle tissue myosatellite cells or those from embryonic or adult stem cells which have the capacity to differentiate into other, specialised cells could be used[61]. "Cells must be cultured in a suitable growth medium, but muscle cells alone cannot produce a muscle. A scaffold is required to provide a structure for cell attachment, and to support cell differentiation and proliferation and permit ingress of blood and other nutrients into the mass of cells. Collagen and fat are also needed in the structure created [62]

d. Examples include that of Atlantic bluefin tuna which is heading towards extinction, but synthetic, cultured fish muscle could replace wild-caught fish and safeguard the species.[63]

we_fyrLbAhXjPZoKHRoYBk4QFghYMAQ&url=https%3A%2F%2Fwww.researchgate.net%2Ffile.PostFileLoader.html%3Fid%3D515568bfd11b8bd22d00000c%26assetKey%3DAS%253A272106741272579%2540144188671164&usg=AOvVaw3jt7vtZlEL4pgePn2Masyu

[60] See https://en.wikipedia.org/wiki/Reptile_scale

[61] Myosatellite cells are the precursors of skeletal muscle cells and differentiate in order to form muscle fibres

[62] Ralph Early (2018) https://www.foodethicscouncil.org/blog/177/19/Is-clean-meat-the-meat-of-the-future/ Accessed 24/05/18

[63] Finless Foods: https://finlessfoods.com/