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Extremophile enzymes for food additives and fertilizers

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The so-called Green Revolution that occurred in the 1950s and 1960s led to landmark increases in agriculture production. The programme included large investments directed towards breeding programmes for cereal crops (wheat, rice and corn) that resulted in high-yielding varieties of plants able to produce more grain. This high productivity required the application of increased amounts of fertilizers and a wider use of pesticides. Also relevant to the increase in production was soil management and the use of heavy machinery.

The green revolution had both positive and negative impacts; for example, the increased agricultural productivity led to a tripling of food production, which helped to prevent or ameliorate famines. One of the negative sides was the environmental impact, with massively increased application of pesticides, that not only killed potential plant pathogens but also many beneficial macro- and microorganisms. Unfortunately, due to the recalcitrance of these xenobiotics, they polluted soils, waters and even food. Also, on the negative side is the overuse of fertilizers that gave rise to leakage of phosphates and inorganic nitrogen that polluted underground waters, surface waters, lagoons and the continental platform of marine environments. This has led to strong eutrophication of many environments that results in reduced biodiversity or even complete destruction of ecosystems.

A current worldwide trend is to develop more sustainable agriculture practices based on environmentally friendly approaches. Within these innovative strategies is the Soil Mission of the European Union which aims to restore more than 70% of currently polluted/degraded soils in the European Union. This particular strategy has promoted the development of living labs that can be used to test new concepts related to sustainable agriculture from small plots to full production scale (Ramos and Lansac, 2020). Microbes play a very relevant role in this process, where they act as plant growth-promoting microorganisms (PGPR). On this level, the aim has been to use microbial consortia and apply them along with the plants, so that the nutrients and environments are balanced correctly for soil restoration. Another way to provide predictability in agri-food production is the use of enzymes – often bacterial – to assist in the mobilization of elements such as phosphorus.

Phosphorus is a macro-element that is found in the cells of all living things. Prokaryotic and eukaryotic cells have developed mechanisms for the transport of inorganic phosphorus from the external environment to the cytoplasm, where it is used in the biosynthesis of phospholipids, sugar phosphates, nucleotides and other molecules (Barea and Richardson, 2015). Despite the fact that phosphorus is one of the most abundant molecules on the earth's surface, it is usually found in non-bioavailable forms, a fact that often leads to phosphorus being a nutrient that limits the growth of plants, bacteria and fungi (Sosa *et al*., 2019). In the soil, inorganic phosphorus is usually solubilized by plants and microorganisms (bacteria and fungi) through the production of a wide variety of weak acids such as citric, lactic, malic, ketoglutaric acid and others (Barea and Richardson, 2015). However, the organophosphorus species in soil and seeds often need to be hydrolysed by phosphatases in order to release inorganic phosphorus. Examples of these species include phytic acid (the main reserve of phosphorus in seeds), sugar phosphates, phospholipids, etc. After they are hydrolysed, orthophosphate is incorporated into the cell through active transport systems (Hayes *et al*., 2000; Tomashow *et al*., 2018). There is considerable evidence that links the phosphatase activity of soils and aquatic systems with the primary productivity of terrestrial ecosystems (Turner *et al*., 2013; Margalef *et al*., 2017) and the primary and secondary productivity of freshwater habitats and marine environments (Martiny *et al*., 2019).

Phosphatases are classified mainly based on their optimal pH, with two groups generically named alkaline phosphatases and acid phosphatases. A series of studies on phosphatases in soils, compiled by Margalef *et al.*

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(2017), revealed that acid phosphatases were present in 329 soil samples compared with 72 observations for alkaline phosphatases. This suggests that acid phosphatases play an important role in the biogenic phosphorus cycle – a role that is not well described for this group of enzymes. Neal *et al*. (2018) and Udaondo *et al*. (2020) defined three families of acid phosphatases, named A, B and C, with class A and C acid phosphatases being well represented among the different microbial taxa.

Alkaline phosphatases and acid phosphatases that release phosphorus from phytate are called phytases (Mullaney and Ullah, 2003). These enzymes are classified as 3-, 5- or 6-phytases depending on where hydrolysis of the ester bond occurs in relation to the carbons of the myoinositol ring. Phytates are of interest for the monogastric animal feed industry because they ease digestibility of grain. Acid phosphatases, including phytases, are also of interest in the detergent and biofuel industry (Vasudevan *et al*., 2019). The use of acid phosphatases in organic agriculture has only recently been explored and represents an underdeveloped area of research. These phosphatases have been used to remove organophosphorus pesticides from the soil (Meng *et al*., 2018), but interest has been expressed towards the development of phosphatases as potential supplements for fertilizers. In fact, our aim is to exploit natural phosphatases and new to nature phosphatases to develop efficient additives for monogastric animals and fertilizers, two relevant applications in the agri-food sector.

Using PROSITE (Sigrist *et al*., 2002), we developed the Pr-GAP, Pr-PhoB and Pr-PhoC profiles, which make it possible for us to identify all three classes of acid phosphatases (Udaondo *et al*., 2020). These profiles have enabled us to identify, among proteins without functional annotation, a set of new acid phosphatases in genomes and metagenomes from soil (including soils contaminated with hydrocarbons from the oil processing industry), compost and marine environments (Udaondo *et al*., 2020). When phosphatases were identified in databases for which DNA samples were not available, we synthesized the corresponding gene with appropriate codon use for expression in *Escherichia coli* (Udaondo *et al*., 2020). Currently, our collection of acid phosphatases includes about 4000 new sequences that were initially not annotated in the databases. By focusing on the set of previously uncharacterized acid phosphatases, we are validating the function of unannotated proteins, and contributing to our understanding of their role in the phosphorus cycle. Among this set of undescribed phosphatases, we have identified an extremophilic acid phosphatases with a number of unexpected properties such as: It remains active in a wide range of pH, i.e. between 3 and 10, although maximal activity was recorded at pH 5.5; an optimal temperature of 55°C but maintaining more than 80% activity at 80°C. These extremophilic properties are relevant to the design of phosphatases that can be used as feed supplements for pigs and chicken and also to design a kind of inorganic fertilizer of the NPK-type in which phosphatases replace P. These phosphatases kept their activity in the presence of high concentrations of inorganic nitrogen and potassium. The molecular basis of this extremophilic phosphatase revealed that the properties derived from a combination of a series of mutations that led to heightened thermodynamic stability. Similar approaches have been carried out in our research group with other enzymes of interest in synthetic biology, for example, to decompose lignocellulosic substrates or synthesis of hydrophobic compounds such as styrene.

These enzymes can be used as partially purified proteins, and added as a component of the production broth or linked to biological membranes so that handling of the enzymes can be adapted to the required application. The use of enzymes for industrial purposes has become very significant since the beginning of this century and we envisage an ample use of enzymes for environmental applications (fertilizers, biodegradation) as well as in the biosynthesis of compounds through the design of novel biosynthetic pathways. Current expectations for gross growth in enzyme use for agricultural is around 8.9% per year from 2020 to 2027 with an ample worldwide expansion (www.industryresearch.com).

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Conflict of interest

None declared.

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