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Review

Autophagy in food biotechnology

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The purpose of this review is not to explain autophagy (as clearly there is a plethora of reviews and research papers on the topic) but to provide the autophagy-savvy reader with an overview of the impact of autophagy research on a number of current topics in food biotechnology. To understand this connection, we need to remember that autophagy is, at the end of the day, a type of stress response. Since as humans we are heterotrophic eukaryotic organisms, our cells, and the cells of those organisms that we consume, use autophagy as part of the day-to-day business of living. Thus, a number of food biotechnology processes such as brewing and winemaking employ eukaryotic organisms under autophagy-inducing conditions, as noted below. In addition, food spoilage processes also involve eukaryotic organisms and these processes also involve physiological aspects that impinge on autophagy. Finally, the recently introduced concept of "functional foods" introduces the possibility of engineering foodstuff for the induction or inhibition of autophagy in the consumer, with a potential promise of health benefits that merits further research.

In this review, we will provide a perspective on the current literature in these three areas, their relationship to current basic research in autophagy, and their future applicative potential.

Autophagy in Food Production Processes

Food and beverage fermentation processes frequently take place in conditions such as prolonged aging steps and/or nutrient limitation that are expected to induce autophagy if eukaryotic microorganisms are involved. Winemaking is an excellent example of these autophagy-prone conditions.[1] Upon inoculation, yeast cells must adapt to the low pH (2.9–3.8) and high sugar concentrations (up to 300 g/l) as well as to the high SO_2 content (40–100 mg/l) before fermentation actually starts. After this lag phase, yeast biomass starts growing exponentially for 2–6 days. The biological activity of the yeast causes various stress conditions throughout the fermentation process, which include rapid nitrogen depletion, temperature variations, and ethanol toxicity. One-third of the ethanol and the main fraction of glycerol present in wine are produced in this step. In the following phase, stationary in terms of yeast biomass increase, the remaining two-thirds of the ethanol and many aromatic compounds which determine wine quality are produced.[2]

Nitrogen limitation is acknowledged to be a major stress factor in this process, preventing growth of *S. cerevisiae* to higher cell densities.[1,3] The most abundant nitrogen sources of grape must are ammonium ions, proline and arginine; the abundance of some other amino acids also can be relevant, depending on the grape variety and agronomic conditions. Proline is usually the most abundant amino acid. However the utilization of proline by yeast can only occur in aerobic environments.[4,5] In winemaking, conditions are generally anaerobic (the denser CO_2 generated by glycolysis expels the other components of air). Even if some oxygen is available during initial stages of fermentation, proline utilization is also blocked by the presence of small amounts of ammonium: ammonium is the preferred nitrogen source for *S. cerevisiae* and an intricate nitrogen catabolite repression mechanism prevents the utilization of proline in the presence of ammonium.[6] Thus, grape must is poor in "yeast available nitrogen" (YAN), and this is the cause for most cases of what the wine industry calls "sluggish or stuck fermentation:" musts that contain insufficient amounts of ammonium and arginine such that the existing biomass does not support a viable rate of fermentation. The wine industry has developed a number of workarounds for this bottleneck, based on the addition of different nitrogen sources, but not devoid of problems, which are beyond the scope of this review. However the bottom line is that grape must is a nitrogen-poor substrate for yeast and that nitrogen starvation conditions prevail during most of the fermentation process. Hence autophagy must also be induced, although no studies have been published to demonstrate this experimentally.

While it is clear that nitrogen starvation conditions occur during grape must fermentation, only one specialized type of fermentation has actually been analyzed with respect to the

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induction of autophagy. In the production of sparkling wines by the "Methode Champenoise," base wine is bottled in specialized bottles with additional sugar and yeast, for a second fermentation. In this second fermentation, even less nitrogen is available, so that CO_2 generation takes place with a relatively low increase in biomass. One important attribute of this step is that autolysis of the yeast takes place after second fermentation, releasing amino acids and other flavor compounds or their precursors into the solution. It has been hypothesized that in winemaking conditions autophagy precedes autolysis, the molecules finally released being the product of a combination of both processes. Cebollero et al.[7] verified one aspect of this hypothesis, by showing that autophagy is induced during the second fermentation. To do this, they used the fact that *atg19*Δ mutants do not have an active Cvt pathway. The Cvt pathway is a specific form of autophagy that occurs in yeast growing in nitrogen-rich medium, and delivers the vacuolar protease aminopeptidase I (Ape1) and α-mannosidase 1 (Ams1) to the lumen of the vacuole without bulk degradation of cytosolic components.[8,9] Atg19 is a receptor protein that recruits Ape1 into the Cvt-pathway.[10] Therefore, *atg19*Δ cells do not deliver prApe1 (62 kDa) to the vacuolar lumen in nitrogen-rich medium. Upon nitrogen starvation and induction of autophagy, Ape1 delivery to the vacuolar lumen occurs due to nonselective recruitment into autophagosomes, leading to the appearance of the mature Ape1 form (50 kDa).[11] By following Ape1 maturation in *atg19*Δ cells, Cebollero et al. showed that autophagy indeed occurs under second fermentation conditions. This finding was further elaborated in a subsequent study,[12] taking advantage of the fact that cytosolic aldehyde dehydrogenase (Ald6p) is a preferred target for autophagy in *S. cerevisiae.*[13] In these studies markers of autophagy appeared far before sugar exhaustion, suggesting nitrogen limitation to be the main inducer of autophagy under these conditions.

Additional support for this hypothesis came when cells overexpressing *CSC1-1*, a dosage-dependent dominant inducer of autophagic sequestration that is an allele of *VPS4,*[14] were shown to undergo drastically increased levels of autolysis under eonological conditions.[15] *VPS4* encodes a AAA-type ATPase that functions in regulating the ESCRT complexes in multivesicular body formation.[16] Conversely, deletion of the *BCY1,* the gene encoding the regulatory subunit of protein kinase A (cAMP-dependent protein kinase) also resulted in accelerated autolysis in these conditions.[17] *BCY1* deletion is pleiotropic, resulting among other phenotypes in impaired autophagy. The apparent paradox is partly explained by the fact that autophagy defective strains die quickly after sugar exhaustion, and would enter autolysis by a different pathway, and in part by the pleiotropic nature of this gene deletion. In light of these studies it was suggested that manipulation of autophagic regulation could lead to increased control over the organoleptic properties of fermented beverages.[18]

Autophagy and Product Shelf Life

Up to approximately 40 percent of foodstuff worldwide is lost due to microbial spoilage. A significant proportion of this loss occurs due to spoilage by growth of unwanted yeast. Yeast tend to spoil foodstuff with high osmolarity, high sugar content, and high acidity.[19,20] Under these conditions, they have a distinct advantage over most bacterial spoilage organisms. However the higher levels of enforcement of effective hygiene standards in food preparation imply a selective disadvantage for bacteria, leading to spreading of food spoilage by yeast in other types of food such as prepared salads and spreads.[20] The most common prey for yeast spoilage, and the industry branch that is hardest hit by losses due to yeast spoilage, however, is the soft drink industry. A fairly wide array of yeast species is found in spoiled food (reviewed in ref. 12). On this list, the commonly used *Saccharomyces cerevisiae* and its sensu stricto brethren have a prominent role as spoilage agents, in a large proportion cases, possibly due to their prevalence in the human biosphere. Due to the fact that yeast are eukaryotic organisms, it is relatively difficult to identify preservatives that will not adversely affect human health. By far, the most common type of preservative used in staving off yeast spoilage are the weak organic acids.

Weak organic acids have been used in food preservation for hundreds of years. For example, benzoic acid was first described as "gum benzoin" by Nostradamus in 1556.[21] The most common weak organic acids used in food preservation include sorbic, benzoic, propionic and acetic acids. Under conditions where the surrounding medium is acidic, to a level around the pK of the acid or lower, a significant amount of the acid molecules will be undissociated and neutral. Under these conditions these molecules are relatively hydrophobic and can associate with, and therefore cross, biological membranes. Once in the cytosol, where the cell maintains a much higher pH (usually around 6–7) the acid dissociates and can no longer cross the cell membrane, effectively being trapped in the cytosol (*S. cerevisiae* cannot metabolize sorbate and benzoate). Thus, as a result of homeostatic efforts of the cell to maintain a distinct environment, the influx of acid into the cytosol is effectively irreversible.[22] This model of action led to the long-standing hypothesis that the cytostatic effects of weak organic acids are due to acidification of the cytosol.[23] Later however, it was found that for the more hydrophobic sorbic and benzoic acids, the minimal concentrations that led to inhibition of cell growth (MIC) had little or no impact on cytosolic pH. In addition, the MIC for benzoate and sorbate are 1–2 orders of magnitude lower than that observed for acetic acid.[24,25] While it is still accepted that hydrophilic acids such as acetic and propionic acid incur growth inhibition through cytoplasmic acidification, these results as well as additional data suggested that benzoic acid and sorbic acid have a different mode of action. Thus, it was also found that specific cellular responses to sorbic and benzoic acids, such as the upregulation of the ABC transporter Pdr12, are specific to acids of carbon chain length 5–8, and not to smaller or larger molecules.[26,27] Additional data suggested that these compounds generated oxidative stress in the cell and it was proposed that benzoic and sorbic acids act through perturbation of intracellular membranes.[22,28]

To gain a better understanding of the effect of weak acids on intracellular membranes, Hazan et al.[29] set out on a more detailed exploration of the effects of these compounds on intracellular membrane trafficking. By comparing the effects of benzoic acid on carboxypeptidase Y trafficking, autophagy, and the Cvt pathway, they concluded that autophagy was completely inhibited by a

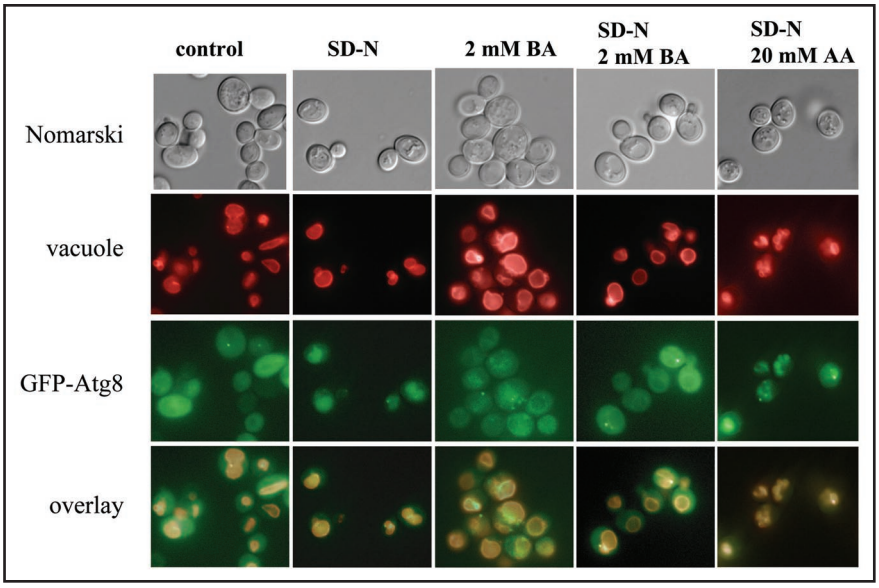

Figure 1. Specificity in the effects of benzoic and acetic acid on trafficking of GFP-Atg8. Yeast cells (*S. cerevisiae*) expressing GFP-Atg8 were stained with 0.8 μM FM4-64 and subjected to starvation, 2 mM benzoic acid, or a combination of starvation and 2 mM benzoic acid as well as starvation plus 20 mM acetic acid. Note that 20 mM acetic acid causes vacuole fragmentation (probably a result of acidification) but does not block delivery of GFP-Atg8 to the vacuole under starvation, in stark contrast to the complete inhibition (but no fragmentation) observed with 2 mM benzoic acid.

concentration of benzoic acid that is commonly found in foodstuff (2 mM), while neither of the other pathways was blocked to this degree. No effect was observed on the Cvt pathway while a kinetic delay was observed in CPY maturation. This selectivity also had a chemical aspect: acetic acid had no effect on autophagy, even at concentrations 1–2 orders of magnitude higher than those used for benzoic acid, while sorbic acid inhibited autophagy at higher concentrations than benzoic acid (see Fig. 1). These results are interesting due to the fact that they suggest that differential perturbation of intracellular membranes may underlie subtle differences in the mechanism of action of these compounds. However a more useful aspect is the fact that inhibition of autophagy implies that benzoic acid treated yeast are hypersensitive to starvation conditions. One major problem with the use of weak organic acids in food preservation is that they are cytostatic, not cytocidal, towards yeast. Hence, yeast tend to adapt to these compounds, eventually achieving levels of growth that are organoleptically noticeable, leading to spoilage.[19] Therefore any treatment that can achieve a cytocidal effect on yeast will tend to prolong shelf life and will therefore have an economic impact. Indeed, Hazan et al. found that benzoic acid, in combination with nitrogen starvation treatment, is cytocidal while either treatment alone is only cytostatic.[29] This result was true both for *S. cerevisiae* as well as for a "professional" food spoilage yeast, *Zygosaccharomyces bailii*.[30]

This synergistic response cannot be directly translated into an improvement in shelf life, as no foods are nitrogen-free. However the discovery by Powers and colleagues[31] that caffeine inhibits Tor in yeast, led to the hypothesis that caffeine, an approved, GRAS food additive, could show the same type of synergism. Indeed, Winter et al.[30] showed that this is the case, both for *S. cerevisiae* as well as for *Z. bailii.* Since the same study also showed that *atg1*Δ cells are caffeine sensitive in and of themselves, the mechanistic implication is that the cellular response to weak organic acids is incompatible with the cellular response to starvation in a way that brings about loss of viability.

Autophagy and Functional Foods

While the first two sections of this review devote space to aspects of food production and preservation, we would like to close by addressing a somewhat fancier issue. The terms functional foods, nutraceuticals, and related expressions were coined in the 1980s and there are no consensus definitions,[32,33] even though legislative bills in several countries do give a definition to some of these nonequivalent terms. The common underlying concept behind these terms is that specific food constituents or ingredients may have specific health benefits beyond basic nutritional functions. These words identify the fastest growing market in the food industry, and a common trend in the regulations governing the marketing of this products in different countries is that "health claims" must be sustained by scientifically sound evidence.[34] Consequently, in addition to its scientific and health interest, improved knowledge of these mechanisms is of economical importance.

There is indeed growing scientific evidence of the identity of food components showing health promoting activity, including molecules from a variety of chemical classes such as peptides, oligosaccharides and phenolics.[35-37] There are also reports on the mechanisms underlying these beneficial effects, which include inhibition of key enzymes (like angiotensin converting enzyme, ACE), and specific cell functions (like sirtuin-mediated transcriptional regulation), among others.

Levels of autophagy decline with age and this has been proposed to underlie a number of aspects of the aging process, among them organelle quality control. For example, the mitochondrial theory of aging states that mutations in the mitochondrial genome accumulate with age and underlie aging phenotypes. As a quality control mechanism, the level of autophagy may determine the kinetics of accumulation of defective mitochondria.[38,39] A related aspect is caloric restriction and its effect on longevity, in which autophagy may also play a role.[38-40] Given these postulated connections between autophagy, aging, longevity, and cancer, many studies have recently focused on the possibility of modulation of autophagy by specific food ingredients.[41]

A growing number of food constituents, including triterpenoids, isothiocyanates, vitamins, trace elements, flavonoids and other phenolics, have been shown to induce autophagy in diverse cell types (recently reviewed by Singletary and Milner[42]). Perhaps the most publicized of these is resveratrol. Resveratrol is a stilbene

found in grape juice, wine, and other plant-derived foodstuff. It is known for its effects on longevity, thought to be caused through an effect on histone acetylation levels.[41,43] More recently, however, resveratrol was shown to induce autophagy in cultured cells and this effect has been suggested to be of physiological relevance.[42] Less well known, but perhaps of equal importance, is the fact that numerous flavonoids, as well as isoflavonoids, protect autophagy from physiological inhibition by a kinase-dependent mechanism.[44] All the above data illustrate the potential of dietary constituents to modulate autophagy in human tissues.

We should be cautious, however, in drawing conclusions about the impact of autophagy modulation by these molecules in human health. A central question is whether, where, and when switching autophagy on or off would be beneficial. We must consider, for example, the multiple functions of autophagy, and the fact that the benefits of cell survival or proliferation will depend on the cell type and pathogenic status; or the requirement of efficient autophagosome clearance in order to avoid disorders due to excess autophagic induction.[43] Researchers studying the role of autophagy in cancer and other diseases agree on the need for further research in order to clarify whether autophagy would be beneficial or pathological in specific disease contexts.[43,44]

One limitation often found on "in vitro" studies about biological effects and mechanism of food constituents is that the doses assayed are generally far from the physiological levels found in human tissues after food ingestion. For example, flavanones, found in plasma at the range of 0.1–2.2 μM after 50 mg aglycone equivalent supply,[45] have shown autophagy promoting activity in the range of 100 μM[42] but there are no studies at lower levels. Some experimental models might also introduce a bias, for example by the use of inhibitors that affect only a part of the autophagy induction pathways. Finally, gastrointestinal digestion, including the activity of gut microbiota and absorption processes, in combination with further biochemical reactions, frequently results in chemical modification of the molecules originally present in foods. All these factors must be taken into account, and relevant studies should consider the actual metabolites found in plasma and internal tissues as well as their physiological concentrations and exposure time. These studies must also cope with the complication that several mechanisms might simultaneously contribute to the biological activity of any given compound or family of compounds (reviewed in ref. 47) as pointed out by Meijer with respect to resveratrol and other compounds.[48]

In conclusion, based on data from epidemiological and intervention studies, the "functional" status of many dietary compounds is undeniable, while data on other molecules are still inconclusive.[46] The mechanisms involved in this biological activity are diverse and are currently the subject of intense research. In this context, modulation of autophagy has recently emerged as one candidate mechanism underlying the activity of several compounds, as illustrated by the examples above. However, carefully designed studies, taking into consideration bioavailability and metabolism of the active molecules, are required in order to understand the true implications of these findings. Thus, much work is still required in order to identify dietary regimes and food components that modulate autophagy in a manner that is relevant for improving human health.

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