

Responses of Yeast Biocontrol Agents to Environmental Stress

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Biological control of postharvest diseases, utilizing wild species and strains of antagonistic yeast species, is a research topic that has received considerable attention in the literature over the past 30 years. In principle, it represents a promising alternative to chemical fungicides for the management of postharvest decay of fruits, vegetables, and grains. A yeast-based biocontrol system is composed of a tritrophic interaction between a host (commodity), a pathogen, and a yeast species, all of which are affected by environmental factors such as temperature, pH, and UV light as well as osmotic and oxidative stresses. Additionally, during the production process, biocontrol agents encounter various severe abiotic stresses that also impact their viability. Therefore, understanding the ecological fitness of the potential yeast biocontrol agents and developing strategies to enhance their stress tolerance are essential to their efficacy and commercial application. The current review provides an overview of the responses of antagonistic yeast species to various environmental stresses, the methods that can be used to improve stress tolerance and efficacy, and the related mechanisms associated with improved stress tolerance.

Postharvest decay of harvested crops caused by fungal pathogens results in significant losses of edible stored fruits, vegetables, and grains. Minimizing these losses is crucial as the worldwide demand for food increases (1). Environmental concerns and food safety issues have made the continued use of chemical fungicides a serious concern, and so several alternative management strategies have been explored. The utilization of wild species and strains of antagonistic yeast species is recognized as one of the promising alternatives. Since Wilson and Wisniewski proposed the first principles and concepts of postharvest biocontrol (2, 3), numerous reviews have been published (4–11). Research has focused on a wide variety of topics. However, the effects of environmental factors on biocontrol systems, especially the viability and efficacy of antagonists, still need to be thoroughly investigated.

Droby et al. (4) highlighted the importance of viewing the use of postharvest biocontrol agents as a tritrophic system involving a plant host, a biocontrol agent, and a pathogen (4). More recently, Liu et al. (8) further reiterated this viewpoint and emphasized that all three of these components are subject to environmental conditions (8). The majority of studies have been conducted on well-characterized strains of the model species such as *Saccharomyces cerevisiae*. Therefore, the information on stress responses derived from these studies may or may not have direct applicability to the wild species and strains of yeasts that have been identified and utilized in postharvest biocontrol research.

Ambient temperature is one of the major environmental stresses experienced by yeast. In *S. cerevisiae* (and likely other related yeasts), the heat shock transcription factor 1 (HSF1) protein family is the primary modulator of the heat shock response (HSR), while a second transcription factor, represented by *Msn2* and *Msn4* genes, also contributes substantially to heat shock gene expression. These transcription factors are responsible for the bulk, if not the entirety, of the HSR (12). Heat shock transcription factors *Msn2* and *Msn4* and the heat shock protein 12 (HSP12) molecular chaperone play an integral role in cold stress responses in yeast species. Importantly, the ability to quickly and efficiently translate genes associated with cold tolerance is a key adaptation in cold-adapted yeasts (13).

As another major environmental stress in biocontrol systems,

oxidative stress causes yeast responses that include an increase in the level of a variety of antioxidants, mediated by complex transcriptional changes. These regulatory mechanisms are aimed at mitigating oxidative injury to cells, such as oxidative damage to DNA, proteins, and lipids. Yeast peroxiredoxin Tsa1 has been recently demonstrated to protect cells from protein-aggregate-induced oxidative stress (14). The adaptive response of yeast species to oxidative stress is largely regulated at the transcriptional level by transcription factors Yap1p, Skn7p, Snt2, Msn2p, and Msn4p. These transcription factors collectively coordinate cellular responses to different oxidative stressors by repressing or upregulating the transcription of specific genes, many of which are associated with antioxidant defenses (15, 16). The process of translation initiation is mediated by a set of highly conserved eukaryotic initiation factors (eIFs) and associated proteins. Translational regulation involves altered protein interactions, activity, and stability, as well as posttranslational modifications (17). More recently, the relocalization of mRNA to mRNA processing bodies (P-bodies) in response to environmental stress in *S. cerevisiae* has been reported to be biphasic. Some mRNAs are present early, whereas others are recruited much later, concomitant with recruitment of translation initiation factors, such as eIF4E (18).

The balance between energy-efficient growth and the ability to rapidly respond to fluctuating environments is a fundamental physiological challenge for all microorganisms, including yeasts. Cellular functions, such as metabolism, stress protection, growth, and proliferation, reflect cell responses to external factors that can

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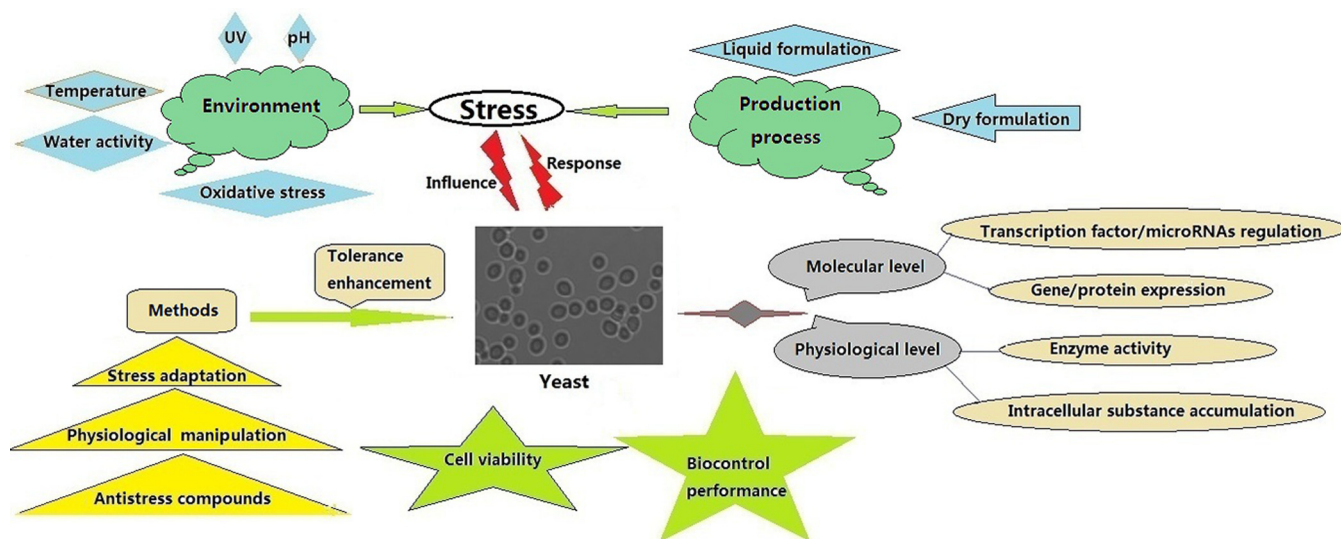


FIG 1 Diagram of responses of yeast antagonists to stresses and methods to improve their tolerance.

be dynamically adjusted to both transient and long-term environmental changes (15, 19). Hershkovitz et al. (20) examined the transcriptomic response of the biocontrol yeast *Metschnikowia fructicola* to grapefruit peel and to the pathogen *Penicillium digitatum* (20). However, no comprehensive studies of the global changes in gene expression in postharvest yeast antagonists in response to abiotic stress have been conducted.

In order for the biological control of postharvest diseases to be successful, antagonists need to possess effective mechanisms to cope with the plethora of abiotic stresses to which they are exposed (5, 21, 22). In this regard, several methods, including physiological manipulation (23, 24), stress adaptation (25, 26), cross-protection (27, 28), and the use of exogenous antistress substances/protectants such as calcium (29), glycine betaine (30, 31), ascorbic acid (32, 33), sugars (33–36), and polyols (37), have been employed to enhance stress tolerance and improve biocontrol efficacy. The purpose of the current review is to provide an overview of research on the role of environmental stresses which affect the viability and performance of antagonistic yeasts. The review focuses on the physiological response of antagonists to stress conditions and on methods that can be used to improve stress tolerance (Fig. 1). Table 1 provides a list of representative studies of responses of antagonistic yeasts to different stresses.

ENVIRONMENTAL STRESSES

Temperature. Once antagonistic yeasts have been applied to a plant surface, exposure to heat stress is inevitable. High temperatures can markedly reduce the viability of biocontrol agents, especially when preharvest applications are administered under field conditions. Some biocontrol yeasts can be administered as preharvest applications (44–46). Therefore, to develop recommendations for their mass production, application, and use, understanding their response to heat stress is essential. Without any protectant, the viability of *Candida oleophila* decreased with increasing temperature (39 to 41°C for 30 min) (27). In contrast, *M. fructicola* exhibited a high degree of thermotolerance, with viability dropping sharply only at temperatures above 43°C (28). More specifically, after a 30-min exposure, the viability of *C. oleophila*

TABLE 1 Representative studies of antagonistic yeasts in response to different stresses

Stress	Yeast species	Reference	
Temperature	Heat	<i>Candida sake</i> CPA-1 38	
		<i>Pichia anomala</i> J121 21	
		<i>Metschnikowia fructicola</i> NRRL Y-27328 28	
	Cold	<i>Candida oleophila</i> I-182 27	
		<i>Debaryomyces hansenii</i> , <i>Pichia membranaefaciens</i> 29	
		<i>Pichia guilliermondii</i> KW-103 36	
	Oxidative stress/low oxygen	ROS	<i>Cryptococcus laurentii</i> LS-28, <i>Rhodotorula glutinis</i> LS-11 39
			<i>Metschnikowia fructicola</i> NRRL Y-27328 28
			<i>Cystofilobasidium infirmominatum</i> PL1 30
		Oxygen limitation	<i>Candida oleophila</i> I-182 27
<i>Pichia caribbica</i> 32			
<i>Pichia anomala</i> J121 40			
<i>Cryptococcus laurentii</i> , <i>Trichosporon pullulans</i> , <i>Rhodotorula glutinis</i> , <i>Trichosporon</i> sp. 41			
Water activity		<i>Pichia anomala</i> J121 21	
		<i>Candida sake</i> CPA-1 38	
		<i>Rhodospiridium paludigenum</i> 42	
pH	<i>Pichia anomala</i> J121 21		
	<i>Candida sake</i> CPA-1 38		
	<i>Rhodospiridium paludigenum</i> 26		
UV	<i>Pichia anomala</i> strain K 43		

cells exposed to 41°C for 30 min dropped to 22%, while the viability of *M. fructicola* cells exposed to 43°C was 87%. Fredlund et al. (21) reported that *Pichia anomala* J121, the yeast used to preserve grains in storage, could grow at temperatures ranging from 3 to 37°C (21). This wide range of temperature tolerance greatly contributed to the competitive ability of this biocontrol yeast strain. An et al. (29) compared the levels of thermotolerance of two other biocontrol yeasts, *Debaryomyces hansenii* and *Pichia membranaefaciens*, and found that the survival of *D. hansenii* exposed to 40°C for 20 min was lower than 10% whereas the viability of *P. membranaefaciens* was approximately 40% after exposure to 50°C for 40 min (29). More recently, the viability of *Pichia guilliermondii* KW-103 was reported to decrease to as low as 4% when it was incubated at 45°C for 15 min (36). Although the induction of HSPs (47), a mitogen-activated protein kinase cascade (48), and other protein kinases (49) involved in heat response was previously reported, intracellular oxidative damage, resulting from the accumulation of reactive oxygen species (ROS) under heat stress conditions, was proposed to play a major role in the decrease of viability. In a study on the antagonistic yeast *Candida sake*, Teixidó et al. (38) did not directly investigate its survival in response to heat stress but provided growth profiles at temperatures ranging from 4 to 37°C in conjunction with low levels of water activity and unfavorable pH (38). Thus, different species and strains of yeast antagonists have various degrees of thermotolerance which make them more or less capable of surviving under different field or packing-house conditions. Different ranges of thermotolerance may also impact efficacy in a manner not directly related to cell viability, albeit this possibility has not yet been addressed in the literature. Vegetables, and especially fruits, are often put into cold storage and a controlled atmosphere in order to extend their commercial shelf life and availability. In the case of fruit crops, the time of storage can extend to up to 10 months. Therefore, the ability of a yeast species to colonize and develop on a host (commodity) at low temperatures is also an important feature. While this aspect of biocontrol systems has not been studied in detail, Vero et al. (50) have explored the use of yeast strains isolated from Antarctic soils as potential biocontrol agents for use under conditions of cold storage (50), and Sangorrín et al. recently reviewed the topic of cold-adapted biocontrol yeasts (51).

Oxidative stress and low oxygen. ROS signaling has been proposed to be a component of the mode of action of yeast antagonists in biocontrol systems. The antagonistic yeasts may serve as an elicitor, triggering ROS signaling in host tissue and leading to the activation of host defenses (52–54). This premise is supported by studies analyzing gene/protein expression during yeast-fruit interactions (20, 55, 56). A proteomic analysis indicated that an antagonistic yeast, *P. membranaefaciens*, induced six antioxidant proteins in peach fruit, including catalase, glutathione peroxidase, and peroxiredoxin. These antioxidant proteins presumably contributed to ROS scavenging, as ROS levels became elevated as a result of pathogen invasion (55). Biocontrol agents must also be able to tolerate ROS-derived oxidative stress, which can affect their viability and efficacy. It has been reported that peroxidases and superoxide dismutase in grapefruit were induced by application of the yeast *M. fructicola*, based on transcriptome analysis (56). Castoria et al. (39) investigated the relationship between oxidative stress resistance and the fitness of the postharvest biocontrol yeasts *Cryptococcus laurentii* LS-28 and *Rhodotorula glutinis* LS-11 (39). *C. laurentii* LS-28, which had higher resistance to

ROS-generated oxidative stress than *R. glutinis* LS-11, exhibited greater colonization and better biocontrol efficacy on apple fruit. It was suggested that oxidative stress resistance could be an important component of the fitness and suitability of a yeast species for use as a postharvest biocontrol agent. That report raised issues about the direct effects of oxidative stress on biocontrol yeasts. In this context, Liu et al. examined the response of *M. fructicola*, *C. oleophila*, and *Cystofilobasidium infirmominiatum* PL1 to oxidative stress (27, 28, 30). They found that *C. infirmominiatum* was the most sensitive to exogenous H₂O₂, while *M. fructicola* was the most tolerant (27, 28, 30). More specifically, survival of *M. fructicola* was 88% in 200 mM H₂O₂, that of *C. oleophila* was 28% in 100 mM H₂O₂, and that of *C. infirmominiatum* was 23% in 20 mM H₂O₂, after a 20-min incubation. Li et al. (32) reported that the survival of *Pichia caribbica* decreased significantly as the concentration of H₂O₂ increased from 5 to 20 mM (32). Almost all of the yeast cells died upon exposure to 20 mM H₂O₂ for 60 min. Thus, in similarity to their thermotolerance characteristics, different yeast species can differ dramatically in their levels of tolerance of oxidative stress.

In addition to oxidative stress, antagonistic yeasts may also have to deal with the low oxygen levels associated with controlled-atmosphere storage. This is especially true for the biocontrol agents used to manage mold in grain stored under airtight conditions (57). In this respect, *P. anomala* J121, a yeast strain used in the biocontrol of mold in grain during storage under conditions of low oxygen and high carbon dioxide, has been extensively studied. *P. anomala* exhibited strong biocontrol of grain mold pathogens such as *Penicillium roqueforti* and *Aspergillus candidus* *in vitro* (58) and also controlled *P. roqueforti* on a variety of grain hosts (59). Studies were conducted on high-moisture-content grains stored under airtight (anaerobic) conditions at both the laboratory scale (57) and the farm scale (60), and the results indicated that *P. anomala* J121 could act as an efficient biocontrol agent (61). Fredlund et al. (40) analyzed growth and metabolite production in *P. anomala* grown under two sets of oxygen-limited conditions: (i) initial aerobic conditions with restricted oxygen access during the growth period and (ii) initial microaerobic conditions followed by anaerobiosis (40). Biomass production was found to be higher under condition i, while the ethanol production rates during growth on glucose under conditions i and ii were similar, indicating that oxygen availability affected respiration but not the fermentation capacity of *P. anomala*. In addition, Tian et al. (41) reported that the concentrations of O₂ and CO₂ affected the viability of four antagonistic yeasts (*Cryptococcus laurentii*, *Trichosporon pullulans*, *Rhodotorula glutinis*, and *Trichosporon* sp.). All of these yeasts, except *R. glutinis*, could even grow well on nutrient yeast dextrose agar (NYDA) after 8 days of incubation at 25°C and at 20% CO₂ (41). The survival of *C. laurentii* was also found to be about 80% after 15 days under controlled-atmosphere conditions (5% O₂ and 5% CO₂) (35).

Water activity. Several attributes of yeast species make them suitable for use as biocontrol agents, including their ability to withstand osmotic stress (8). Many antagonistic yeast species can grow under conditions of low water activity (*a_w*) (21). Once a yeast species has been applied to a commodity, *a_w* depends on the environmental conditions within the host. For example, cereal grain is commonly harvested with a water content of 20% to 22%, corresponding to *a_w* 0.92 to 0.95. When glycerol was used as an osmolyte, *P. anomala* was able to grow at *a_w* 0.85 both in a liquid

and on a solid substrate. In contrast, the yeast was unable to grow on a substrate containing NaCl below a_w 0.92, which emphasizes the difference between a compatible solute and a salt solution (21). The growth of *C. sake* was also evaluated in nutrient yeast dextrose broth (NYDB) supplemented with an ionic solute, NaCl, and a nonionic solute, glycerol, in the a_w range of 0.995 to 0.85. Results indicated that the minimum a_w values for sustaining yeast growth were 0.92 with NaCl and 0.90 with glycerol. Both *P. anomala* and *C. sake* could grow at a lower a_w when glycerol was used as an osmolyte rather than NaCl. Importantly, since the studies were performed in a nutrient-rich growth medium, the responses of a yeast species to low a_w could be quite different in a minimal-growth medium or under the limited-nutrient conditions found on the intact surfaces of grains, fruits, and other plant surfaces.

As growth at low a_w is advantageous for biocontrol activity, yeasts isolated from high-osmosis environments, such as the ocean, pickled juices, soy sauces, or honey, may be a good source of new antagonists (8). The ability of some yeasts to grow in these salt solutions led to the idea of combining yeast antagonists with various salts, such as CaCl_2 , MgCl_2 , and NaHCO_3 (62, 63), which has now become a fairly common approach of enhancing biocontrol efficacy, as yeasts appear to be more tolerant of the low a_w and the component salt ions. The effect of different solutes (NaCl, glycerol, and glucose) on the growth of *Rhodospiridium paludigenum*, an antagonistic yeast isolated from the south of East China Sea, was previously examined (42). Results indicated that low water activity ($a_w = 0.98, 0.97, 0.96,$ and 0.95) inhibited the growth of *R. paludigenum* in NYDB but that the yeast grew better in a medium supplemented with NaCl solute than with other nonionic solutes (glycerol and glucose). *R. paludigenum* grown in 6.6% NaCl-modified medium had higher viability (92.1%) under conditions of low water activity ($a_w = 0.95$) than the control (81.1%) after a 48-h incubation. Salt-adapted *R. paludigenum* also showed higher tolerance of freeze stress and exhibited better biocontrol performance on pears and jujubes than the nonadapted culture. Overall, the ability of a yeast antagonist to grow at a low water potential may represent an important attribute regarding its use as a biocontrol agent and so should be considered when selecting and developing a potential biocontrol strain.

Other environmental factors. When antagonists occupy a plant host (fruit, vegetable, or grain), the host pH either in the macroenvironment or in the microenvironment can have a direct impact on growth and establishment. *C. sake* is tolerant of a wide pH range (3–7), regardless of a_w (38). Liu et al. (27) reported that oxidative-stress-adapted *C. oleophila* grew faster in liquid culture at pH 4.0 (the same pH value as that used to produce Golden Delicious apple flesh at commercial maturity) than non-stress-adapted yeast (27). The *in vitro* growth of the stress-adapted yeast at pH 4.0 correlated well with *in vivo* growth and resulted in greater efficacy in inhibiting apple rots caused by *P. expansum* than non-stress-adapted yeast. Wang et al. (26) assayed low pH tolerance in *R. paludigenum* and found that preexposure of the yeast to pH 4.0 or 5.5 for 36 h of incubation, adjusted with malic acid or lactic acid, improved the survival of the yeast when it was subsequently exposed to a lethal pH of 1.0 to 3.0 for 1 h (26). The preexposure to pH 4.0 or 5.5 also resulted in a higher growth rate on apple and greater biocontrol efficacy.

UV-B radiation (280 to 320 nm) is also a major environmental factor that can affect the viability of biocontrol agents when they

are applied under field conditions. The effect of UV-B radiation on *Pichia anomala* strain K was evaluated *in vitro* and *in vivo* on apple fruit (43). To achieve lethal doses of 50% and 90%, respectively, UV-B at 0.89 and 1.6 KJ/m^2 or at 3.2 and 5.76 KJ/m^2 had to be applied *in vitro* or *in vivo*. Cosupplementation of *P. anomala* with several protectants, such as riboflavin, folic acid, gelatin, lignin, and tyrosine, was shown to be effective in reducing yeast mortality caused by UV-B radiation.

EXPOSURE TO STRESS DURING PRODUCTION

A commercial biocontrol product needs to have a formulation that provides adequate shelf life while retaining efficacy. Both dry and liquid formulations have been used for the production of commercial biocontrol products. During the production process, biocontrol agents are exposed to severe abiotic stresses that can have a profound effect on their viability and subsequent performance. Sugars, skim milk, and “antistress” substances are often utilized as protectants to ameliorate production-related stresses. In general, dry formulations, compared to wet formulations, offer the advantage of longer shelf life without the need for refrigeration as well as protection from contamination and greater convenience when the product is shipped and distributed (35, 64).

Dry formulation. Freeze-drying is one of the most common methods used to obtain dry preparations of microorganisms (65, 66). It was reported that freeze-drying at -20°C using 10% skim milk as a protectant was the best method to preserve the viability of yeast (*C. sake*) cells (34). Freezing cells in liquid nitrogen resulted in the greatest injury to cells, reducing viability to $<10\%$. The viability, efficacy, and stability of freeze-dried *C. sake* were further tested using different protective and rehydration media (67). The highest level of biocontrol activity was obtained when lactose and skim milk (10% lactose–10% skim milk) were used as protectants during the freeze-drying process and 1% peptone was added when cells were rehydrated prior to use. The use of nutrient protectants at those high concentrations, however, is not economically feasible for large-scale production. Melin et al. (64) compared different formulations (using freeze-drying, vacuum drying, fluidized bed drying, and liquid formulation) of *P. anomala* J121 and found that the best viability was obtained by freeze-drying the yeast (64). The initial viability after freeze-drying was as high as 80% when trehalose was used as a protectant during the freeze-drying process. Freeze-drying was also tested on *Cryptococcus laurentii* and *Rhodotorula glutinis*, and both the endogenous and exogenous levels of trehalose were found to play a critical role in enhancing cell viability (35, 68).

Freeze-drying is relatively expensive compared to other production methods and requires special equipment for batch drying. Therefore, many studies have explored alternative drying methods for producing biocontrol agents, including spray drying, fluidized bed drying, and vacuum drying. Abadias et al. (69) indicated that spray drying of *C. sake* resulted in a very high level of cell damage, thus greatly reducing viability (69). Although a mild heat treatment could induce thermotolerance, it did not improve the survival of yeast (*C. sake*) cells exposed to spray drying enough for the method to be considered a suitable approach to commercial production (70). Fluidized bed drying and vacuum drying, along with freeze-drying and liquid formulations, of the yeast antagonist *P. anomala* J121 have also been evaluated (71). A shelf life of a few months could be obtained, regardless of which production method was used. The use of fluidized bed drying required the

addition of cottonseed flour as the main carrier ingredient during the drying process. This method of drying was also evaluated using the yeast-like fungus *Aureobasidium pullulans* (72). *A. pullulans* (Ach 1-1) was grown in a glucose-fed batch fermentor for 48 h. The cells were then dried in a fluidized bed dryer to obtain a viability level of 62%. Although viability declined with time, 28% of the initial viability was observed after 7 months when the material was stored at 4°C. Vacuum drying is similar to freeze-drying except that elevated drying temperatures and more-moderate vacuum levels are utilized. Cells are maintained in a nonfrozen state during the whole drying process and thus are metabolically active well into the drying process (73). The addition of trehalose, and the polymer polyvinylpyrrolidone, when vacuum drying the biocontrol agent, *P. anomala* J121, resulted in high levels of viability and a shelf life of at least 6 months at room temperature (64).

Liquid formulation. The use of liquid media is another commonly employed method of producing biocontrol agents for commercial use. Liquid formulations have been extensively evaluated in the use of bacterial biocontrol agents such as *Pseudomonas fluorescens* (74) and *Bacillus* spp. (75) but not so extensively in the use of yeast-based biocontrol products. In the case of *C. sake* CPA-1, different media, temperatures, and levels of water activity have been evaluated in relation to a liquid formulation (76). Results indicated that a shelf life of approximately 7 months could be obtained without a significant loss in viability or efficacy when *C. sake* cells were grown in a sorbitol-modified medium and then stored in an isotonic solution of trehalose. In another pilot-scale study, production of a liquid formulation of *Rhodotorula minuta* was evaluated (77). The yeast was formulated at 10⁹ CFU/ml in a phosphate buffer solution, with the addition of glycerol (20%) and xanthan (0.5%). The additives greatly inhibited contamination of the final product and prevented cell sedimentation. Using this formulation, a cell count of 10⁷ CFU/ml could be maintained for up to 6 months when the formulated product was stored at 4°C. The maintenance of the viability of *P. anomala* in a liquid medium amended with lactose, starch, or trehalose was evaluated (78). Supplementing the storage medium with either lactose or trehalose allowed a high level of viability to be maintained over an 8-to-12-week period at all the temperatures tested (−20 to 30°C). Oxidative stress is one of the major factors leading to a decrease in the viability of yeast cells stored in a liquid formulation (77, 79). On the basis of this premise, *L*-ascorbic acid was added in combination with sugar protectants (trehalose and galactose) to enhance the viability and efficacy of *Cryptococcus laurentii* and *Pichia membranaefaciens* in a liquid formulation (33). The shelf life could be extended to 90 days at 4°C and to 15 days at 25°C. Table 2 provides a list of formulation studies conducted on antagonistic yeasts.

IMPROVING STRESS TOLERANCE AND BIOCONTROL EFFICACY

Methods for improving stress tolerance in antagonistic yeasts include preadaptation (acclimation) to a stress, physiological manipulation, and the addition of antistress compounds to either the growth medium or the storage medium. Few studies have utilized a transgenic approach to enhancement of stress tolerance due to regulatory and consumer concerns. Currently, the added value provided by transgenic approaches simply does not warrant the time or expense of pursuing this approach, except as an academic pursuit to better understand the molecular basis of stress tolerance.

TABLE 2 Representative formulation studies of antagonistic yeasts

Formulation and yeast species	Reference(s)
Dry	
Freeze-drying	
<i>Candida sake</i> CPA-1	34
<i>Pichia anomala</i> J121	64, 71
<i>Cryptococcus laurentii</i> , <i>Rhodotorula glutinis</i>	35, 68
Spray drying	
<i>Candida sake</i> CPA-1	69
Fluidized bed drying	
<i>Pichia anomala</i> J121	71
<i>Aureobasidium pullulans</i> (yeast-like fungus)	72
Vacuum drying	
<i>Pichia anomala</i> J121	64, 71
Liquid	
<i>Candida sake</i> CPA-1	76
<i>Rhodotorula minuta</i>	77
<i>Pichia anomala</i> J121	71, 78
<i>Cryptococcus laurentii</i> , <i>Pichia membranaefaciens</i>	33

Stress adaptation. Stress adaptation, i.e., exposing an organism to a mild stress in order to increase its tolerance of a much stronger stress, has been reported to induce cross-protection against a variety of abiotic stresses in plants (80, 81) and against prokaryotic and eukaryotic microorganisms (82–84). Specifically regarding yeast antagonists, changes in gene expression during heat stress/oxidative stress adaptation and a subsequent improvement in stress tolerance and biocontrol efficacy have been reported (27, 28). A mild heat shock pretreatment (40°C, 30 min) improved the tolerance of *M. fructicola* to subsequent high-temperature stress (45°C, 20 to 30 min) and oxidative stress (0.4 mM H₂O₂, 20 to 60 min), at least partially due to the induction of trehalose-6-phosphate synthase 1 (TPS1) expression and the accumulation of trehalose (28). The role of TPS1 in abiotic stress tolerance has also been characterized in two model yeast species, *S. cerevisiae* (85) and *Schizosaccharomyces pombe* (86). Pretreatment of *C. oleophila* with 5 mM H₂O₂ for 30 min increased its tolerance of a subsequent severe oxidative stress (50 mM H₂O₂), high temperature (40°C), and low pH (pH 4) by activation of a cellular antioxidant system (27). In addition to heat stress and oxidative-stress adaptation, preexposure to a low pH has been recently reported to improve the viability of *R. paludigenum* exposed to a subsequent lethal pH (26). Pretreatment with mild concentrations of salt also improved the tolerance of freezing stress and the biocontrol efficacy of *R. paludigenum* (42). Similar results were obtained in bacterial species used for biocontrol, where preadaptation to osmotic stress was used to enhance the biocontrol efficacy of *Bacillus subtilis* and *Brevibacillus* sp. against *Fusarium* head blight in wheat (87).

Physiological manipulation. Manipulation of the concentration of intracellular polyols and sugars may be a good approach for improving the ecological fitness of yeast antagonists under field conditions. In research conducted with *C. sake*, the culture medium (NYDB) was supplemented with glycerol, glucose, trehalose, NaCl, sorbitol, or proline to adjust the level of water activity (*a_w*) and the intracellular concentration of polyols and sugars (23, 37). This resulted in improved ecological fitness and abiotic stress tolerance. Similarly, physiological manipulation was also successfully used on *P. anomala* to enhance its biocontrol efficacy and to

reduce ochratoxin A contamination by *Penicillium verrucosum* (24). Yeast cells were grown in a liquid, molasses-based medium supplemented with proline to create different levels of a_w (0.98 and 0.96), which resulted in a significant (up to 50%) increase in the concentrations of trehalose and arabinol.

Antistress compounds. Sugars, polyols, and skim milk are commonly used in formulations of biocontrol agents to help maintain viability during the production process, including liquid culture (33, 78, 88), freeze-drying (34, 35, 67, 68), spray drying (69, 70), vacuum drying (64), and fluidized bed drying (71). Among sugar protectants, trehalose, a nonreducing disaccharide, is generally accepted to function as a protective metabolite. The protective function of trehalose is based on the water replacement hypothesis or the glass transition hypothesis (89, 90). Trehalose allows the intracellular solution to undergo a phase transition (becoming glass-like), to remain in disequilibrium with the external vapor pressure, in response to dehydration caused by drying or freezing. The glass-like structure of the cytoplasm prevents cellular injury resulting from the increased concentrations of salts and other potentially toxic compounds and also prevents membranes from adhering to each other. Thus, the plasma membrane is protected and lipid peroxidation is reduced (35, 91). In addition to trehalose, other disaccharides (sucrose and lactose), monosaccharides (glucose, fructose, and galactose), and trisaccharides (raffinose) have also been evaluated for their protective effects (34). Glucose provided a highly significant (over 90%) protective effect on cell viability in *Pichia guilliermondii* exposed to 45°C (36). Ascorbic acid, as an antioxidant, has been applied alone (32) or in combination with various sugars (33) to enhance oxidative stress tolerance and biocontrol efficacy. Glycine betaine (*N,N,N*-trimethyl glycine), a compatible solute, not only acts as an osmoprotectant but also induces an antioxidant response in biocontrol yeasts at either the transcriptional level (*Candida oleophila* [31]) or the enzymatic level (*Cystofilobasidium infirmominiatum* [30]).

CONCLUSIONS AND FUTURE OUTLOOK

The commercial use of wild species or strains of yeasts as biocontrol agents presents unique challenges in terms of production and product stability. Most initial studies with biocontrol agents utilized high-cost nutrient media that are not economically feasible for large-scale production. Producing a product that has a long shelf life and maintains efficacy also represents a significant challenge. Although the effects of culture conditions on the physiology and stress tolerance of model yeasts have been extensively studied, much more research is needed to assess how much of the currently available information is applicable to the production of a wide array of natural, wild species and strains of yeasts. Such information may need to be generated on a case-by-case basis, especially for the use and production of “exotic” yeast species. The past 30 years of research have produced an exponential increase in the number of yeast species that have been identified as potential biocontrol agents for the management of postharvest diseases. The desire to circumvent the hurdles to commercialization of these yeasts is providing the impetus for increased research efforts to better understand how these novel yeast species respond to abiotic stress.

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