Nutrient-Dependent Efficacy of the Antifungal Protein YvgO Correlates to Cellular Proliferation Rate in *Candida albicans* 3153A and *Byssochlamys fulva* H25

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Abstract YvgO is a recently characterized antifungal protein isolated from *Bacillus thuringiensis* SF361 that exhibits a broad spectrum of activity and pH stability. Customized colorimetric metabolic assays based on standard broth microdilution techniques were used to determine the variable tolerance of *Byssochlamys fulva* H25 and *Candida albicans* 3153A to YvgO exposure under select matrix conditions impacting cellular proliferation. Normalization of the solution pH after antifungal challenge expanded the available pH range under consideration allowing for a comprehensive in vitro assessment of YvgO efficacy. Indicator susceptibility was examined across an array of elementary growth-modifying conditions, including media pH, incubation temperature, ionic strength, and carbohydrate supplementation. Under suboptimal temperature and pH conditions, the indicator growth rate reduced, and YvgO-mediated susceptibility was attenuated. While YvgO association but not efficacy was somewhat influenced by solution ionic strength, carbohydrate supplementation was shown to be the most influential susceptibility factor, particularly for *C. albicans*. Although the specific choice of carbohydrate/nutrient supplement dictated the extent of enhanced YvgO efficacy, D-glucose additionally improved the association between antifungal and target. Indeed, when exposed to YvgO under conditions that lead to increased cellular proliferation, both indicators displayed a stronger association and susceptibility to YvgO when compared to carbohydrate-deprived media or suboptimal incubation environments. With further study, YvgO may have the capacity to function as a prophylaxis for food safety and preservation, as well as a pharmaceutical agent against opportunistic fungal pathogens either independently or in combination with other established treatments applied to both livestock and human health concerns.

Keywords Fungal inhibition · XTT assay · Heat-resistant mold · Human fungal pathogen

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Introduction

Current antifungal methodologies for the clinical and food safety fields are limited due to low therapeutic thresholds, poor stability or efficacy, and increasing resistance [11, 14, 24]. As with antibacterial compounds, the need for new antifungal compounds is becoming more relevant as clinical and food-processing challenges continue to escalate. In addition to producing the bacteriocin Thurincin H [30], *Bacillus thuringiensis* SF361 produces and secretes YvgO, a novel proteinaceous agent showing select antifungal activity toward several genera of filamentous fungi including *Aspergillus*, *Byssochlamys*, and *Penicillium* as well as the pathogenic yeast *Candida albicans* [20]. To gain a better understanding of the biological activity of YvgO, two primary indicators, *Byssochlamys fulva* H25 and *C. albicans* 3153A, were selected due to their high susceptibility, their relevance to either food or clinical applications, and their dissimilar morphology.

In our previous work, we reported that YvgO is stable for at least 2 weeks at physiological and ambient temperatures and is neither acid nor alkali labile, surviving for at least 2 weeks in solutions ranging from pH 3 through 12 [20]. In this initial study, after incubation, the activity of YvgO was assessed through the dilution of titers upon solid nutrient agar, rather than directly at the incubation test conditions. Up to this point, the functionality of YvgO at variable matrix composition or solution pH is unknown. As such, the aims of the current study were threefold: (1) determine the differential efficacy of YvgO against the chosen indicators under variable solution pH, nutrient supplementation, and other growth-modifying conditions, (2) accomplish the previous goal using a straightforward analytical method that can be readily adapted for proteinaceous and variable pH systems, and (3) correlate specific indicator response metrics to YvgO efficacy in an effort to elucidate potential means of biological activity.

To meet objective one, the choice and style of assessment outlined in objective two becomes critical. In regards to the second objective, standardized broth dilution methods do provide defined guidelines for determining breakpoint parameters and quality control measures but are only validated for select antifungal agents against a limited set of indicators [2]. Since we are interested in YvgO's effect upon select indicators under specific growth conditions, we will need to be free from such narrow constraints and have instead opted for an assay that gives a more detailed response to antifungal activity. Similar to other colorimetric susceptibility assays [16, 18], tetrazolium salt-based methods are essentially a broth microdilution assay utilizing a colorimetric indicator for the quantitative comparison of antimicrobial susceptibility, providing a more detailed assessment over measurements of titers, diffusion techniques, or broth dilution methods [4, 7, 13, 21] while also suitably comparing to established standardized broth dilution methods [5, 6] outlined by the Clinical and Laboratory Standards Institute (CLSI). To that end, an assay involving the reduction of XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) [23] has been optimized and modified to best assess the cytotoxic efficacy of YvgO throughout an extended range of matrix considerations against both model indicators. In addition, this assay will provide essential quantitative measures of EC_{50}, YvgO/indicator association, and minimum inhibitory concentration (MIC), permitting a more thorough characterization of YvgO efficacy. This study does not attempt to redefine standard antimicrobial testing procedures. Our methodology requires the use of modified media formulations and growth conditions that would need to deviate from prescribed CLSI approaches. Also, as we are not comparing the efficacy of YvgO against other antifungal compounds, standard visual end point CLSI approaches to antimicrobial testing have been used for comparative and validation purposes only.

During the initial characterization of such a novel antifungal compound with diverse applications ranging from pharmaceutical agent to food protectant, developing an understanding of its biological activity against sensitive fungal strains is required. To initiate this line of research, variable YvgO susceptibility was quantified based on the influence of select matrix conditions typical of physiological and food-based systems that affect indicator growth, which as will be shown, affect YvgO efficacy.

Materials and Methods

Microbial Indicators, Media, and YvgO Antifungal Protein Production

Two microorganisms of differential morphology but elevated susceptibility were chosen to serve as the indicators used to assess YvgO efficacy. A *B. fulva* H25 spore crop (10^5 spores/ml) was prepared and stored as described previously [20]. *C. albicans* 3153A was maintained on potato dextrose agar (3.9 % PDA, BD Difco; Franklin Lakes, NJ USA) acidified to pH 3.5 with 10 % tartaric acid. To develop an active culture, a single colony was transferred to 5 mls of tryptic soy broth (3 % TSB, BD Difco; Franklin Lakes, NJ USA) and incubated for 16 h at 30 °C. These media were chosen as they each can be simply modified as needed to exploit potential differences in efficacy observed upon each indicator being studied.

The semi-purified (>95 % purity) ammonium sulfate precipitation fraction (40 % saturation of the supernatant adjusted to pH 6) of YvgO was prepared as previously described [20], resuspended in Milli-Q water (Millipore Corporation; Bedford, MA USA), and generated within 1 day prior to use. The absence of synergistic or complementary activity in the semi-purified preparations was verified against HPLC-purified YvgO.

Susceptibility assays were carried out using several media (BD Difco; Franklin Lakes, NJ USA): [1] CAA, 3.0 % Casamino acids supplemented with 1.5 % yeast extract, [2] PDB, 2.4 % potato dextrose broth; and [3] TSB, 3.0 % tryptic soy broth. Precise pH alterations and/or carbohydrate supplementation were made as necessary. The media for all optimizations and assays were filter sterilized through a 0.2-µm polyethersulfone (PES) membrane (Millipore Corporation; Bedford, MA USA).

XTT and Menadione

The XTT assay typically functions better when the reduction of the XTT substrate occurs in the presence of an

electron-coupling reagent such as menadione (22). XTT (Sigma-Aldrich; ST. Louis, MO USA) was dissolved in Milli-Q water to a final working concentration of 2 mg/ml. A 25 mM solution of menadione (Sigma-Aldrich; ST. Louis, MO USA) was prepared directly in acetone and diluted in Milli-Q water to a final working concentration of 250 μM. Each solution was filtered through a 0.2-μm PES membrane and stored in amber vials at 4 °C. Equal volumes of each component was warmed to room temperature and combined immediately prior to use.

Experimental Design and Optimization of XTT Parameters

Assays were carried out in triplicate using sterile 96-well flat bottom polystyrene microtiter plates (Costar; Corning, NY USA) and analyzed using an MRXTC Revelation microtiter plate reader (Dynex Technologies, Chantilly, VA USA) set at 490 nm for XTT/menadione absorbance and 600 nm for XTT-independent monitoring of fungal cell growth and calculation of cellular proliferation rates. While incubations for all stages of optimization were initially examined at both 30 and 37 °C, subsequent optimized analyses were carried out at 30 °C.

During XTT assay optimization, initial indicator densities were varied from 10^2 to 10^5 cfu/ml, while XTT and menadione concentrations were assessed from 25 to 200 μg/ml and 0 to 125 μM, respectively. Indicator growth periods and post XTT incubation periods were optimized based on the differential growth rate between each indicator. In order to assess the amount of growth after XTT/menadione addition all wells were examined visually and at 600 nm in an XTT-independent fashion. In doing so, there is a direct comparison between the quantitative XTT assay and a visual method of detection dictated by the CLSI [4, 21, 26]. Incubations for the optimizations were performed with CAA media adjusted to pH 6.

The optimized XTT assay consisted of 150 μl aliquot of 10^5 cfu/ml indicator combined with 50 μl of the twofold serially diluted antifungal preparation. Fifty microliters of sterile water in place of the antifungal served as the unrestricted growth control (theoretical maximum), while sterile media in place of the inoculant served as the negative growth control (theoretical minimum). *B. fulva* spores were incubated at 30 °C for 24 h, while *C. albicans* was incubated at 30 °C for 6 h. Following incubation, 50 μl of XTT/menadione was added (final concentrations 200 μg/ml, 25 μM, respectively) and incubated for 2 h (*B. fulva*) or 30 min (*C. albicans*). Plates were read after a 15 min incubation following a 25 μl 1 M phosphate buffer addition, described below.

Effect of Matrix pH on the Reliability and Feasibility of the XTT Assay

The complication with assessing a pH-variable system with tetrazolium salts is twofold: (1) the absorbance from reduced XTT is maximal at pH 6.2, decreasing substantially at lower pH values and (2) the background absorbance due to matrix interference is unacceptably high at elevated pH. To adapt the technique to suit the current study's needs and expand the general utility of the assay, the pH of the base media was varied integrally from 3 to 10 to assess the growth rate of each indicator and determine the pH-sensitive effectiveness of XTT/menadione. The indicator growth as measured at 600 nm, matrix background absorbance, and XTT-generated signal of each pH-adjusted media was compared against a second set of wells receiving 25 μl of 1 M phosphate buffer (pH 6.2), added either before or after XTT/menadione incubation. Duplicate XTT-free wells were inspected visually and also measured at 600 nm as an XTT-independent assessment of fungal growth and YvgO susceptibility. This led to the final optimized procedure described in the previous section.

YvgO Efficacy as Dictated by Incubation Media pH

Due to growth restrictions, the pH-dependent susceptibility of each indicator was determined integrally from pH 3 to 8 in CAA media. The starting concentration of the YvgO dilution series was 25 μg/ml and 1,600 μg/mL for *B. fulva* and *C. albicans*, respectively. It was determined experimentally that the pH of each well did not vary significantly due to antifungal addition or indicator growth during the assay time span.

YvgO Efficacy due to Media and Carbohydrate Supplement

For each indicator, three primary media were used to assess susceptibility: (1) CAA, (2) PDB, and (3) TSB, as described earlier. In addition, both indicators were tested against CAA media supplemented with either 5 or 20 g/L of either D-glucose or mannose (Fisher Scientific; Pittsburgh, PA USA). Additionally, each of these formulations were tested integrally from pH 3 to 8.

YvgO Efficacy due to Altered Solution Ionic Strength

In a similar fashion, the base CAA media was supplemented with increasing concentrations of sodium chloride (Fisher Scientific; Pittsburgh, PA USA). The ionic strength of the media was varied from 25 to 200 mM. Throughout this range, indicator susceptibility was assessed at pH 3.5,

6.0, and 7.4 in the presence or absence of 20 g/L D-glucose or mannose.

Data Analysis and Statistical Interpretation

Indicator proliferation was described as normalized percent growth, the maximum defined as the YvgO-free control. The fungi-free wells served as the zero-signal background. The sigmoidal curves generated from the YvgO-mediated growth response were analyzed using a nonlinear, four parameter E_{max} regression model [8–10] with a variable slope and no preset constrictions on sigmoidal curve minimums or maximums, described by the equation:

$$y = a + (b - a)/(1 + 10^{((x-c)*d)}) \tag{1}$$

where "a" is the theoretical minimum of the sigmoidal curve; "b" is its theoretical maximum; "c" is the log EC_{50} (the concentration of antifungal required to reduce growth by 50 % from the theoretical maximum); and "d" is the slope, a convenient measure of the strength of association between the indicator and YvgO. As mathematically described, the slope would generate a negative value. However, for clarity, all slopes are given as absolute values.

Sigmoidal curves were described as an average of three independent experiments each performed in triplicate, with a standard deviation about the mean. EC_{50} and slopes were statistically described using the 95 % confidence interval (CI) range. The lowest concentration of YvgO restricting growth to no more than 5 % over the background control was defined as the MIC. These were additionally confirmed visually using simple broth dilution methods. Treatment comparisons were performed using one-way analysis of variance (ANOVA) and the Tukey's HSD test. Correlations between solution pH, susceptibility within data sets, and between YvgO efficacy and cellular proliferation were described using Spearman's rank correlation test.

Results and Discussion

There were no significant differences during optimization or subsequent treatments *within* trials incubated at 30 and 37 °C. Therefore, for ease of explanation, and to ensure that hyphal growth in *C. albicans* is prevented, all presented data reflects the 30 °C incubation trials. However, when compared between 30 and 37 °C, elevated incubation temperatures resulted in decreased indicator doubling times, resulting in decreased EC_{50} and MIC values. Details for the XTT optimization protocol follow the YvgO/ microbe interaction study.
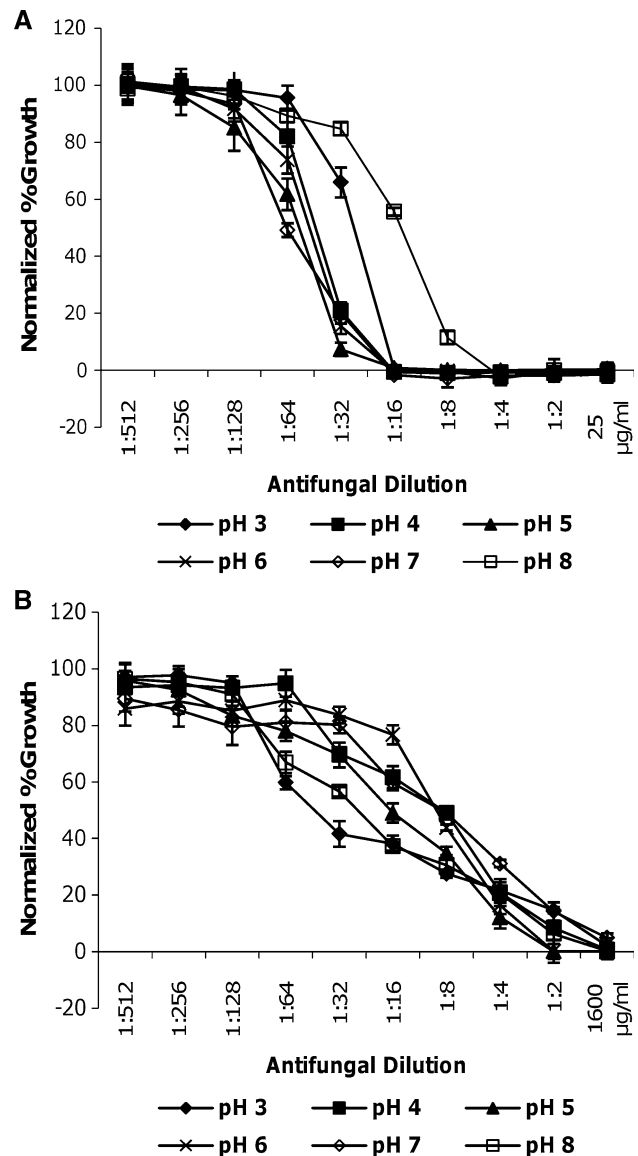

Fig. 1 XTT-determined growth curves as influenced by incubation media pH generated by *B. fulva* H25 (**a**) and *C. albicans* 3153A (**b**) exposed to serially diluted additions of the YvgO antifungal protein. Each trial was performed using CAA as the only comparative media. All points are presented as a percentage of growth normalized from a theoretical maximum taken from control samples lacking YvgO antifungal. Data points are means of triplicate trials given with standard deviations about the mean

YvgO Efficacy as Dictated by Incubation Media pH

Figure 1 (*B. fulva*, top; *C. albicans*, bottom) depicts the graphical illustration of the YvgO-mediated susceptibility of each indicator from pH 3 to 8, using non-supplemented CAA. Illustrations such as this offer a visual assessment of both the efficacy (EC_{50} and MIC) and antifungal/indicator association (slope). The corresponding quantitative account of these parameters is found at the top of Tables 1 (*B. fulva*) and 2 (*C. albicans*). *B. fulva* response shows

Table 1 Nonlinear regression analysis of *B. fulva* H25 susceptibility to YvgO under variable nutrient conditions

Media	Condition	Visual MIC[a] (μg/ml)	XTT MIC (μg/ml)	EC$_{50}$[b] (95 % CI[c], μg/ml)	Slope (95 % CI, absolute value)
CAA[d], no supplement	pH 3	3.13	1.56	0.864 (0.837–0.892)	2.153 (1.991–2.314)
	pH 4	3.13	1.56	0.570 (0.564–0.575)	1.227 (1.194–1.259)
	pH 5	3.13	1.56	0.446 (0.391–0.508)	1.145 (0.877–1.414)
	pH 6	3.13	1.56	0.514 (0.459–0.576)	1.158 (0.943–1.373)
	pH 7	3.13	1.56	0.411 (0.392–0.431)	0.775 (0.706–0.844)
	pH 8	6.25	6.25	1.700 (1.675–1.726)	0.882 (0.834–0.931)
CAA + 20 g/L mannose	pH 3	3.13	1.56	0.614 (0.570–0.662)	1.941 (1.430–2.453)
	pH 4	3.13	1.56	0.551 (0.434–0.700)	2.077 (1.671–2.483)
	pH 5	3.13	1.56	0.581 (0.482–0.700)	2.637 (2.310–2.964)
	pH 6	3.13	1.56	0.531 (0.509–0.555)	1.665 (1.487–1.843)
	pH 7	3.13	6.25	0.762 (0.568–1.024)	1.105 (0.480–1.550)
	pH 8	6.25	6.25	0.607 (0.586–0.628)	0.617 (0.578–0.657)
PDB[e], no supplement	pH 3	3.13	3.13	0.536 (0.399–0.719)	0.572 (0.310–0.833)
	pH 4	1.56	1.56	0.851 (0.569–1.274)	1.614 (1.202–2.027)
	pH 5	1.56	1.56	0.636 (0.483–0.837)	0.888 (0.622–1.154)
	pH 6	1.56	1.56	0.745 (0.721–0.771)	1.562 (1.439–1.685)
	pH 7	1.56	1.56	1.025 (0.937–1.121)	3.994 (3.394–4.594)
	pH 8	3.13	1.56	0.669 (0.597–0.749)	1.019 (0.934–1.105)

All values represent the average of three independent experiments each performed in triplicate, with a standard deviation about the mean

[a] Minimum inhibitory concentrations (MIC) were taken as the last dilution of antifungal to show no appreciable increase (5 %) in cell growth over cell-free controls

[b] EC$_{50}$ = effective concentration of antifungal required to reduce cell growth by 50 %

[c] CI = confidence interval

[d] CAA = Casamino acids minimal media as described in text

[e] PDB = standard potato dextrose agar

significant deviations in EC$_{50}$ at pH 8 ($\alpha = 0.01$) and pH 3 ($\alpha = 0.05$). This increase in pH was correlated ($\alpha = 0.05$) with decreasing YvgO association, which was further correlated ($\alpha = 0.05$) to decreases in cellular proliferation rate. While *C. albicans* failed to show pH-mediated differences in susceptibility, significantly decreased slopes ($\alpha = 0.05$) and MICs ($\alpha = 0.01$) were observed when compared to *B. fulva*.

YvgO Efficacy Due to Media and Carbohydrate Supplement

Variations in carbohydrate concentration were not significantly different. Tables 1 and 2 also describes the nonlinear regression analysis for *B. fulva* and *C. albicans*, respectively, as dictated by media supplementation. *B. fulva* incubated in PDB or CAA media supplemented with glucose or mannose (both 5 and 20 g/L) had no statistically significant changes in susceptibility over non-supplemented controls at any pH tested. As these supplements did not appreciably alter the growth rate of *B. fulva*, a correlation between cellular proliferation and antifungal

concentration was not observed. Furthermore, when incubated in carbohydrate-rich media, the previously observed correlation between media pH and antifungal association was lost, most likely due to observed normalized growth rates.

The data sets for *C. albicans* are more informative. There is a distinct alteration in both YvgO efficacy and association as the carbohydrate source was varied. The base CAA media lacking a carbohydrate supplement displayed the poorest YvgO efficacy (Fig. 1b) along with the poorest cellular proliferation rate. Mannose-supplemented media increased the efficacy approximately fourfold, while the association between the antifungal and yeast remained unchanged. There was a far better increase in YvgO association when the CAA media was supplemented with D-glucose. A visual interpretation of the differences between the mannose supplement (Fig. 2a) and the D-glucose supplement (Fig. 2b) is noteworthy. However, the strongest association and efficacy against *C. albicans* was observed with PDB (Fig. 2c). In this case, the EC$_{50}$ and MIC decreased significantly ($\alpha = 0.01$) while the slopes were improved over non-supplemented and mannose-

Table 2 Nonlinear regression analysis of *C. albicans* 3153A susceptibility to YvgO under variable nutrient conditions

Media	Condition	Visual MIC [a] (μg/ml)	XTT MIC (μg/ml)	EC$_{50}$ [b] (95 % CI [c], μg/ml)	Slope (95 % CI, absolute value)
CAA [d], no supplement	pH 3	1,600	1,600	66.46 (51.38–85.96)	0.280 (0.232–0.329)
	pH 4	1,600	1,600	199.7 (185.0–215.6)	0.345 (0.316–0.374)
	pH 5	800	800	130.9 (115.6–148.1)	0.308 (0.280–0.337)
	pH 6	800	800	207.1 (192.7–222.5)	0.698 (0.626–0.770)
	pH 7	1,600	1,600	304.5 (230.7–401.9)	0.275 (0.235–0.316)
	pH 8	1,600	1,600	68.18 (60.86–76.37)	0.265 (0.243–0.287)
CAA + 20 g/L mannose	pH 3	800	800	48.23 (44.22–52.62)	0.316 (0.282–0.349)
	pH 4	400	200	18.37 (15.35–21.99)	0.338 (0.296–0.380)
	pH 5	200	200	17.94 (15.66–20.54)	0.350 (0.324–0.377)
	pH 6	200	200	40.04 (17.93–42.26)	0.558 (0.506–0.610)
	pH 7	200	200	23.68 (21.61–25.94)	0.562 (0.518–0.606)
	pH 8	200	200	14.84 (13.15–16.76)	0.517 (0.404–0.630)
CAA + 20 g/L D-glucose	pH 3	50	50	17.01 (16.68–17.36)	1.920 (1.818–2.021)
	pH 4	100	50	23.80 (22.09–25.68)	0.796 (0.692–0.901)
	pH 5	100	100	37.88 (28.97–49.53)	0.544 (0.375–0.712)
	pH 6	100	100	48.06 (46.56–49.61)	0.852 (0.782–0.922)
	pH 7	100	100	51.25 (50.00–52.53)	1.549 (1.461–1.636)
	pH 8	100	100	36.95 (34.88–39.15)	0.959 (0.888–1.029)
PDB [e], no supplement	pH 3	50	50	19.12 (15.84–23.08)	0.698 (0.554–0.841)
	pH 4	50	50	22.43 (21.66–23.21)	1.197 (1.120–1.273)
	pH 5	25	25	8.372 (5.485–11.78)	0.651 (0.450–0.852)
	pH 6	25	25	10.56 (6.478–17.20)	0.708 (0.450–0.966)
	pH 7	25	25	12.57 (9.556–16.53)	0.891 (0.616–1.166)
	pH 8	25	25	9.957 (7.677–12.91)	1.394 (0.322–2.466)

All values represent the average of three independent experiments each performed in triplicate, with a standard deviation about the mean

[a] Minimum inhibitory concentrations (MIC) were taken as the last dilution of antifungal to show no appreciable increase (5 %) in cell growth over cell-free controls

[b] EC$_{50}$ = effective concentration of antifungal required to reduce cell growth by 50 %

[c] CI = confidence interval

[d] CAA = Casamino acids minimal media as described in text

[e] PDB = standard potato dextrose agar

supplemented CAA (α = 0.01). Not surprisingly, a correlation between efficacy and proliferation rate is observed (α = 0.01) as the growth rate of *C. albicans* increases throughout this series. Interestingly, TSB (commercially formulated with 5 g/L glucose) displayed similar efficacy to CAA supplemented with glucose, while dextrose-supplemented CAA did not perform as well as PDB (commercially formulated with 20 g/L dextrose).

In effect, the most efficacious response to YvgO occurred when solution conditions favored rapid indicator proliferation. In *B. fulva*, at higher pH values, growth was impeded and the efficacy of YvgO diminished, even though the association between YvgO and indicator remained generally unchanged. When offered a media with a carbohydrate source as opposed to a starvation-style media, *C. albicans* grew markedly faster, was more strongly

associated with YvgO, and became more susceptible. While mannose conferred a faster cellular proliferation rate that correlated with increased susceptibility, the addition of the preferentially metabolized D-glucose [28] resulted in the most robust proliferation rate and strongest antifungal efficacy. This mirrors other lines of research indicating that glucose, as opposed to mannose, is primarily responsible for cell growth, metabolism and other regulatory factors in many strains of yeast [17, 19, 25].

Ionic Strength Considerations

Along with changes in solution pH, the ionic strength of the surrounding matrix can influence the electrostatic interactions between a protein antagonist such as YvgO and the cell surface of its intended target [1, 12]. For toxins that
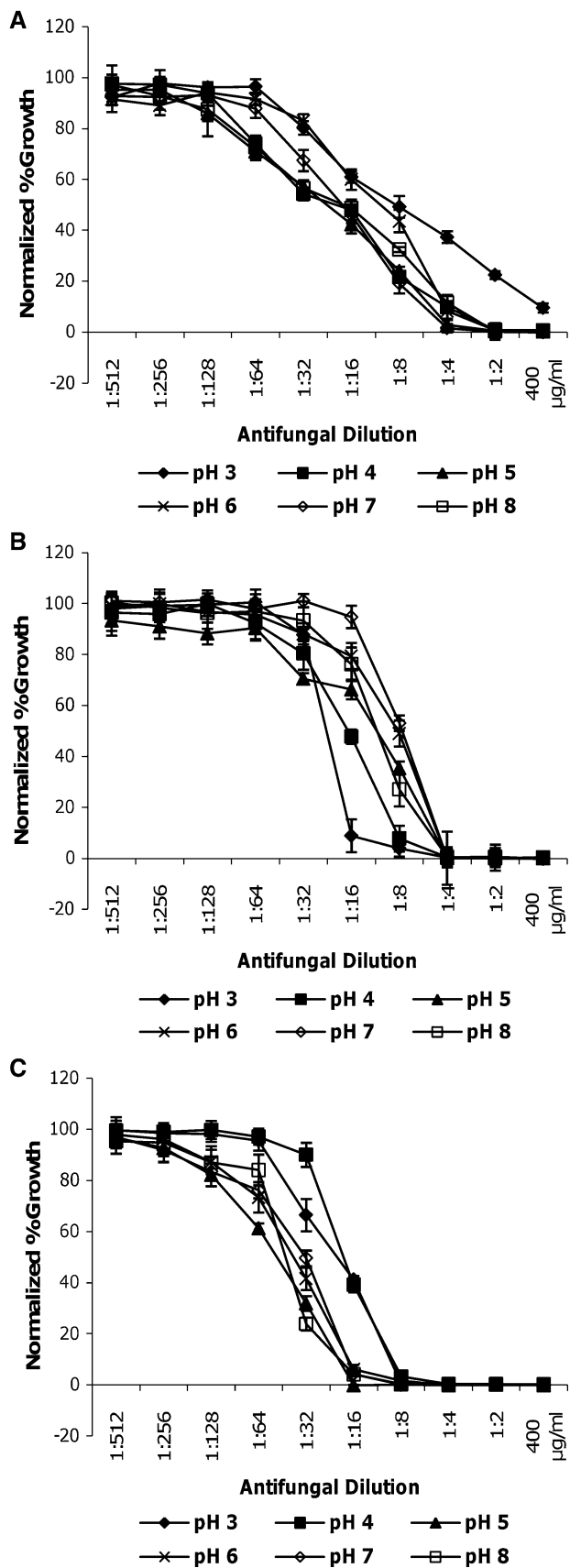
◄**Fig. 2** XTT-determined growth curves as influenced by alterations in media and carbohydrate supplement generated by *C. albicans* 3153A exposed to serially diluted additions of the YvgO antifungal protein. **a** CAA supplemented with 20 g/L mannose; **b** CAA supplemented with 20 g/L D-glucose; **c** potato dextrose broth (PDB). All points are presented as a percentage of growth normalized from a theoretical maximum taken from control samples lacking YvgO antifungal. Data points are the average of three independent experiments each performed in triplicate, with a standard deviation about the mean

utilize such an initial mode of attachment, disrupting this process would subsequently affect the adsorption and toxicity to the target cell. Assessments were difficult above an ionic strength >100 mM as indicator growth suffered and required increased incubation time to achieve reliable results. Media tested at physiologically relevant conditions of pH and ionic strength resulted in a similar efficacy against each indicator. At pH 6, there were no statistically relevant alterations in EC_{50} or MIC values when comparing indicator susceptibility in media of varying ionic strength, with or without carbohydrate supplementation. Regardless of carbohydrate supplementation, under acidic conditions (pH 3.5), an increased ionic strength resulted in a reduced rate of growth, which in turn, resulted in moderately reduced slopes ($\alpha = 0.05$). In general, increasing ionic strength typically resulted in a decreased association between YvgO and its target (i.e., decreased slope). As this could have consequences for cell wall attachment or internalization, several lines of inquiry are currently underway.

XTT Optimization for pH-Variable Matrices

The XTT metabolic assay provided a great deal of information relevant for this study but suffered from a limited utility. In order to expand the pH range and better reflect the varied chemistries seen in food-based matrices, a pH modification step needed to be added and thoroughly validated. Furthermore, the assay needed to be validated for the novel use of a proteinaceous antimicrobial agent with a potentially pH-sensitive efficacy depending upon the toxin's pK_a and its ability to interact with specific microbial indicators. The initial optimization strategy was adapted from work performed with amphotericin B and itraconazole against specific *Aspergillus* spp. [22]. An analysis of the indicator density, XTT/menadione concentration, and incubation times that led to the described optimized method is provided in supplemental Figures S1, S2, and S3. The specific conditions chosen (Fig. 3) conferred the strongest signals and lowest standard deviations for the current study. The optimization protocol, when repeated with other proteinaceous antimicrobials (i.e., bacteriocins) against different indicators (i.e., bacilli, lactobacilli), is easily customized for alternate toxin/microbe combinations

Fig. 3 Optimized XTT method parameters for YvgO-based efficacy trials against *B. fulva* H25 and *C. albicans* 3153A. The *top* shows the concurrent optimization of XTT and menadione response to *B. fulva* (**a**) or *C. albicans* (**b**) indicator. The *bottom* illustrates the response of the idealized XTT concentration across varying concentrations of menadione and initial spore/cell densities for *B. fulva* (**c**) and *C. albicans* (**d**). These trials utilized optimized indicator incubation times of 24 and 6 h for *B. fulva* and *C. albicans*, respectively, and XTT/menadione exposure incubation times of 120 and 30 min for *B. fulva* and *C. albicans*, respectively. *Shaded* areas indicate the chosen parameters used in the current study. The complete optimization protocol can be found by referring to supplemental Figures. 1–3
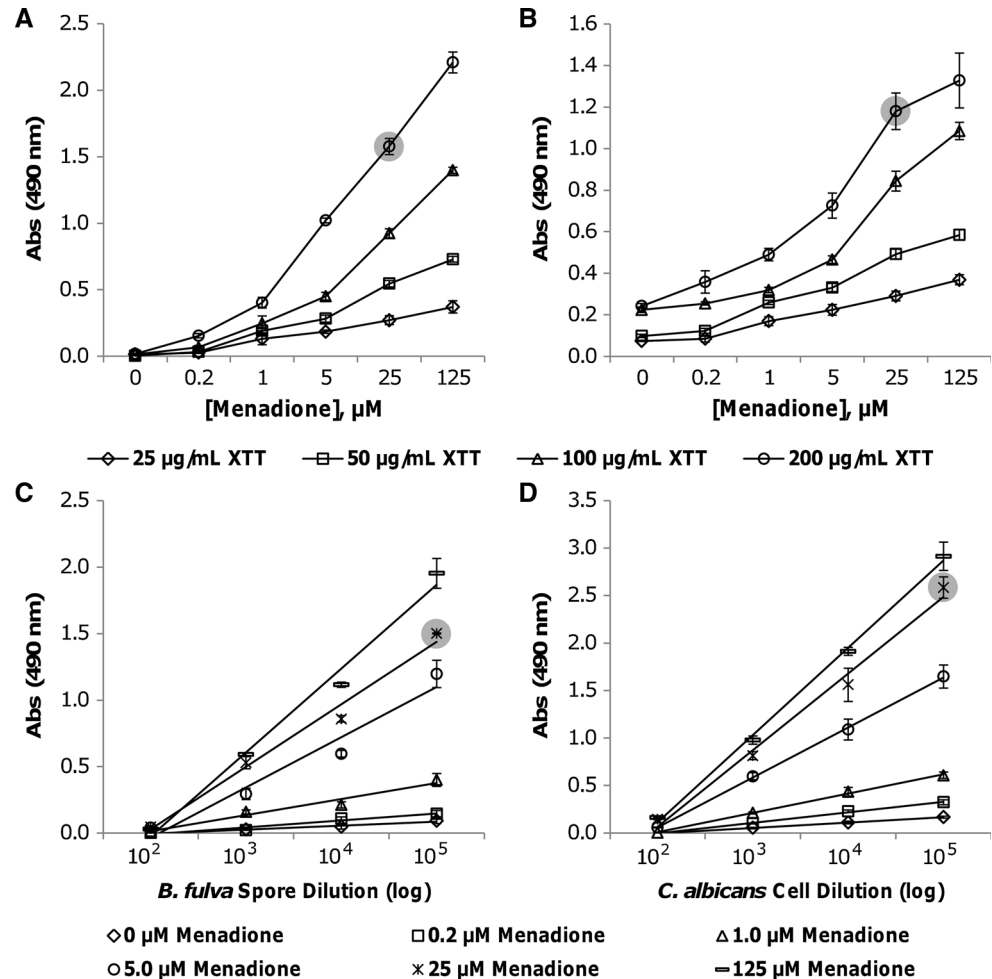
and under various physiological and food-based matrices. This utility is further expanded with post incubation modification of solution pH, as described below.

At lower pH values, reduced XTT is protonated and fails to give an appreciable signal at any visible wavelength (Fig. 4a, *B. fulva*; c, *C. albicans*) even though there is sufficient indicator growth up to pH 8 (Fig. 4b, *B. fulva*; d, *C. albicans*). At a higher pH, the increased matrix background signal becomes problematic. While the pH buffer can be introduced either prior to or after XTT/menadione addition, the timing of the addition was not important as each resulted in a complete recovery of XTT absorbance, regardless of initial incubation pH (Fig. 4, right). In this fashion, XTT can be a reliable indicator of the remaining cell population after YvgO exposure, while normalizing the reduced XTT signal to its maximum absorbance. Furthermore, under these conditions the signal due to XTT absorbance was statistically similar to the indicator growth determined visually by simple broth dilution methods. Therefore, from a method development standpoint, this study offers the added benefit of expanding the use of the

XTT assay as a means for the rapid metabolic screening of several quantitative metrics of antimicrobial efficacy, including in vitro testing at variable matrix pH (Fig. 4).

Concluding Remarks

The biological activity of YvgO appears similar to other cytotoxic compounds that exploit upregulated mechanisms of cellular metabolism and growth observed in rapidly proliferating cells. The relationship between cellular proliferation and cytotoxicity is well documented in areas concerning chemotherapy and cell cycle metabolism [29].

Already shown to be adaptable to fungi [22] and yeast such as *Candida* spp. [3, 15], using the modifications to the XTT assay, a direct quantification of the proliferation-dependent efficacy of YvgO across all viable solution pHs is described. As the XTT assay relies on metabolically active cells to measure cytotoxicity, a strong correlation between YvgO efficacy and cellular proliferation can be established. The expansion of this assay to include a wider

Fig. 4 XTT assay optimization for pH-variable matrices. **a** *B. fulva* H25 and **c** *C. albicans* 3153A: The problem. The *dark bars* indicate the background absorbance of cell-free media supplemented with the optimized concentrations of XTT and menadione. The *light gray bars* indicate the XTT absorbance due to cell growth. The *traced line* shows the unacceptably decreasing usable signal under the varying pH conditions. **b** *B. fulva* and **d** *C. albicans*: The solution. The *dark bars* now represent background-corrected unbuffered signal generated by XTT reduction by either *B. fulva* H25 (**b**) or *C. albicans* (**d**) across the pH range from 3 to 10. The *light gray* bars, also background-corrected, show the same conditions as altered by the addition of the pH 6.2 buffer prior to spectroscopic analysis. The *traced line* shows the cellular growth as read via spectroscopic absorbance at 600 nm
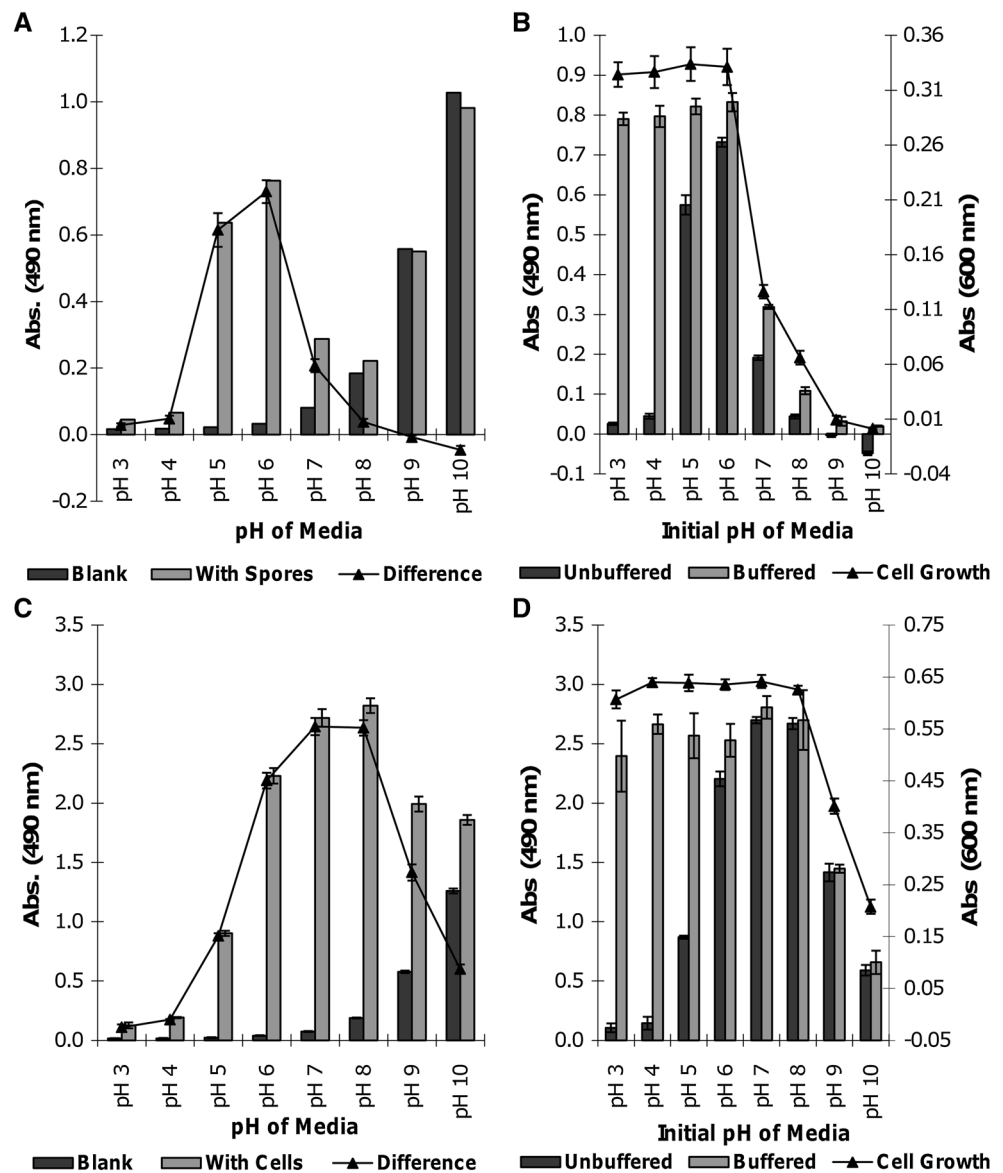
pH range will be of interest to the fruit juice and beverage industries where heat-resistant molds are often a concern in various acidic commodities [27].

This study offers the first considerations concerning the biological activity for YvgO based on cellular proliferation correlations. As a complement to these initial studies, collaborative work to elucidate YvgO's tertiary structure based on 3D NMR analysis in order to compare structure–activity relationships is forthcoming. Widening the search for natural antimicrobials and preservatives is imperative. Proteinaceous compounds of this sort offer unique selectivity that may be exploited in several scientific disciplines. Due to its robust nature and efficacy, clinical and industrial applications for the YvgO protein are promising. In the food industry, YvgO could see significance in liquid-based commodities that suffer from spoilage to due heat-resistant molds such as *B. fulva* or contamination from mycotoxin producing molds such as *Penicillium* spp. and *Aspergillus* spp. As YvgO is efficacious in model physiological solutions, an inquiry into potential cross-sensitivity to mammalian cell lines and synergistic interactions with established antifungal pharmaceuticals is currently underway. Furthermore, in an effort to reduce the effective dosage of relatively toxic antifungal pharmaceuticals, the role of YvgO as a potential complementary cofactor for established antifungal therapies is also being explored.

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