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Growth Inhibition by Glucose Oxidase of Selected Organisms Associated With the Microbial Spoilage of Shrimp *(Pandalus jordani)*: In Vitro Model Studies

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ABSTRACT

The effect of glucose oxidase (GOX)-catalase with different GOX-catalase ratios and of the chemicals produced by GOX, gluconic acid and hydrogen peroxide, were evaluated on four microorganisms usually present in shrimp. Growth inhibition was independent of the enzyme ratio within the limits tested. Hydrogen peroxide inhibited *Pseudomonas fluorescens*, *Hansenula polymorpha* and *Acinetobacter calcoaceticum*. Only *Corynebacter aquaticum* was inhibited by gluconic acid but was not affected by hydrogen peroxide.

Glucose oxidase (E.C. 1.1.3.4 β-D-glucose:oxygen oxidoreductase) (GOX) catalyzes the oxidation of glucose to gluconic acid with the production of hydrogen peroxide. Enzymatic preparations contain catalase (CAT) that decomposes H_2O_2 to H_2O and O_2 (*1,21*). Glucose oxidase is used to stabilize foods against problems arising from the presence of oxygen and glucose and for the production of gluconic acid. Egg albumin, egg yolk, whole egg, dried meat, and potatoes are desugared to prevent browning and off-odors caused by Maillard reactions (*16,19*). Available oxygen is scavenged from orange juice concentrates, syrups, and carbonated citrus-flavored soft drinks to maintain freshness and color (*20*).

The use of glucose oxidase-catalase has been proposed as an on-board preservation system for seafood (*9-11,22,26*). Field (*10*) showed that GOX/CAT used as a dip, incorporated in ice or immobilized on an algin blanket, extended by 67% the shelf life of whole and filleted winter flounder. Shaw et al. (*22*) evaluated the use of GOX/CAT to prolong the freshness of iced cod fillets and postulated oxygen removal by the oxidation of glucose by GOX/CAT as the likely mode of action. Field (*10*) had suggested a lower surface pH as the cause for the preservative effect, although application of gluconic acid showed no effect on the shelf life of cod fillets (*22*). Dondero et al. (*9*) demonstrated a great potential of GOX/CAT as an immersion system for the on-board preservation of shrimp (*Heterocarpus reedi*). In spite of nearly neutral pH values, they observed significant preservative effects.

The predominant microorganisms in freshly caught Pacific shrimp are *Moraxella*, *Pseudomonas*, *Acinetobacter*, *Arthrobacter*, and *Flavobacterium-Cytophaga* spp. (*15*). In Gulf Coast shrimp, Cobb et al. (*8*) found *Vibrio*, *Pseudomonas*, and/or *Moraxella-Acinetobacter* spp. to be the initially dominant species. After 12 to 15 d of ice storage, *Vibrio* spp. disappeared and *Pseudomonas* and *Moraxella-Acinetobacter* spp. predominated. The first drip from the ice contained *Moraxella-Acinetobacter*, *Pseudomonas*, *Flavobacterium*, *Micrococcus*, *Vibrio*, *Corynebacterium*, *Staphylococcus*, *Alcaligenes* spp., and yeasts. After 9 to 13 d, *Vibrio*, *Flavobacterium*, and *Micrococcus* spp. could not be detected, whereas *Pseudomonas* and *Moraxella-Acinetobacter* spp. remained or increased in number (*8*). A recent study on the microflora of Georgia Coast brown shrimp (*Penaeus aztecus*) reported *Acinetobacter*, *Enterobacter*, and *Flavobacterium* spp. predominant in fresh shrimp and *Acinetobacter* and *Flavobacterium* spp. predominant after 18 d of ice storage (*13*). In general, cold-water fish carry a predominantly gram-negative population (*Moraxella*, *Acinetobacter*, *Flavobacterium* and *Vibrio* spp.) (*23*). A significant occurrence of coryneform bacteria and micrococci has also been reported (*24*). Aside from pseudomonads, other frequently isolated but usually minor components of the spoilage flora include *Moraxella*, *Acinetobacter*, *Brochothrix thermosphacta*, and *Alteromonas putrefaciens*. The spoilage potential of these organisms is only manifest under restricted conditions where the growth of *Pseudomonas* spp. is suppressed (*12*). Coryneform bacteria may persist at levels in excess of 10% of total population, but micrococci are present in only small numbers (*24*).

In addition to bacteria, yeasts and occasionally fungi are reported to occur on fish and shellfish. Pagnocca et al. (*18*), working with shrimp (*Penaeus schmitti*) off the Brazilian coast, found yeast populations on the order of 10^2 CFU/g that included *Hansenula* spp. Although there is less information available on these microorganisms, there is little or no evidence to suggest an important role for either yeast or fungi in changes that occur in stored fish and shellfish (*24*). There are reports indicating that a psychro-

philic pink yeast may have been responsible for the spoilage of stored oysters (*24*). These differences notwithstanding, spoilage patterns during iced storage are usually quite similar and are mainly caused by *Pseudomonas* spp. and *Alteromonas putrefaciens* (*2,14*).

The objective of this study was to evaluate the effectiveness of GOX/CAT solutions with different GOX/CAT ratios on the growth inhibition of four microorganisms usually found in shrimp. The GOX/CAT mode of action on the inhibition of these microorganisms (*Pseudomonas fluorescens*, *Acinetobacter calcoaceticum*, *Corynebacter aquaticum*, and *Hansenula polymorpha)* was also investigated.

MATERIALS AND METHODS

Reagents

Glucose oxidase (Catalog No. G7141, from *Aspergillus niger*, 138 U/mg with 4 U/mg protein of catalase) (GOX) and catalase (Catalog No. C10, from bovine liver, 5000 U/mg protein) (CAT) were purchased from Sigma Chemical Co. (St. Louis, MO). Nutrient broth was from Difco (Detroit, MI). Hydrogen peroxide, glucose, gluconic acid, and other analytical reagents were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Microorganisms

Pseudomonas fluorescens ATCC 15456 and *Acinetobacter calcoaceticum* ATCC 14601 were obtained from the American Type Culture Collection (Rockville, MD). Additional organisms were isolated from freshly caught commercial shrimp. Samples (11 g) were blended for 1 min in a Waring blender with 99 ml sterile 0.1% peptone water. Appropriate dilutions prepared in the same diluent were spread plated on plate count agar (PCA, Difco) and incubated at 7°C for 10 d. Distinctive colonies were picked at random from countable plates, spread on PCA plates and similarly incubated. After several transfers, two strains showed significant growth in nutrient broth rehydrated in seawater (SNB). Using fatty acid analysis, they were identified as *Corynebacterium aquaticum* and *Hansenula polymorpha* (Five Star Laboratory, Branford, CT).

The four selected microorganisms were grown at 24°C in nutrient broth prepared in artificial seawater (*3*) (Table 1) using a rotary shaker (New Brunswick Scientific, Edison, NJ) set at 175 rpm. Growth was monitored spectrophotometrically at 550 nm (Spectronic 20, Bausch and Lomb, Inc., Rochester, NY). When mid-exponential phase was reached, the culture was centrifuged, and the pellet formed was resuspended in nutrient broth containing 17% glycerol. Aliquots (5 ml) were kept frozen at -80°C until needed. The number of viable microorganisms was determined the day before use by spread plating on PCA and incubating at 24°C for 24 h.

TABLE 1. *Composition of artificial seawater.*

Compound	g/kg of soln
NaCl	23.926
Na_2SO_4	4.008
KCl	0.677
$NaHCO_3$	0.196
KBr	0.098
H_3BO_3	0.026
NaF	0.003
$MgCl_2$	5.600
$CaCl_2$	1.110
Distilled water to 1000 g	

Source: Bidwell & Spotte (*3*).

Growth inhibition studies

Media preparation. Nutrient broth medium was rehydrated with phosphate buffer (9.25 g KH_2PO_4 + 5.71 g Na_2HPO_4 dissolved in 1 L of artificial seawater, pH 6.5) (SNBP) to determine the effect of GOX/CAT ratio, and with artificial seawater only to determine the effect of gluconic acid and hydrogen peroxide. Aliquots (50 ml) were added to 300-ml side-arm flasks and autoclaved at 121°C for 15 min. Seventy grams of glucose was dissolved in 100 g of distilled water and filtered sterilized (0.20 µm cellulose nitrate membrane, Nalge Co., Rochester, NY); 5.5 g of this solution was added to the heat sterilized nutrient broth to obtain a 4% glucose concentration.

Effect of GOX/CAT ratio. Duplicate side-arm flasks with SNBP inoculated with 10^4 or 10^2 CFU/ml of the appropriate microorganism were incubated at 24°C. The GOX/CAT ratios used were 35:1, 5:1, and 1:1 when expressed as activity units measured at standard conditions (*1*); the GOX level was kept constant at 1 U/ml (32 mg was dissolved in 20 ml of distilled water and 0.25 ml added to each side-arm flask). The GOX/CAT 35:1 was the glucose oxidase preparation and catalase contamination declared by the manufacturer. The GOX/CAT 5:1 treatment was prepared by dissolving 50 mg of catalase in 250 ml of distilled water; this solution was diluted 1:10 and 0.1 ml added to the corresponding side-arm flasks. For the GOX/CAT 1:1 treatments, 0.5 ml of the diluted catalase solution was added. Side-arm flasks with 10^4 CFU/ml were immediately treated with GOX and CAT. Side-arm flasks with 10^2 CFU/ml were treated with GOX and CAT only after reaching exponential phase. Uninoculated side-arm flasks with the appropriate GOX/CAT ratio were used as blanks, while inoculated nutrient broth in seawater served as controls.

Effect of gluconic acid and hydrogen peroxide. Duplicate side-arm flasks were incubated at 24°C with constant shaking (175 rpm). Treatments are shown in Table 2. Uninoculated SNB with the different treatments were used as blanks for the absorbance measurements. GOX/CAT was added to positive controls when the exponential phase was reached. The amount of gluconic acid added was determined by constructing a curve relating pH of a glucose-glucose oxidase solution to gluconic acid production and by monitoring pH change in the positive control. Gluconic acid, hydrogen peroxide, and the two in combination were added in amounts equal to that produced in the positive control over the previous 90 min, until no further decrease in pH was observed in the positive control. The addition of hydrogen peroxide followed a 1:1 molar ratio with gluconic acid as indicated by the following equation:

$$\text{GLUCOSE} + O_2 + H_2O \xrightarrow{\text{GOX}} \text{GLUCONIC ACID} + H_2O_2$$

TABLE 2. *Treatments used to assess the effect of gluconic acid and hydrogen peroxide on the growth of test microorganisms.*

Treatment	Description
Untreated control	Inoculated SNB[a]
Positive control	Inoculated SNB + GOX/CAT[b]
Gluconic acid effect	Inoculated SNB + GA[b] (periodic addition)
Hydrogen peroxide effect	Inoculated SNB + H_2O_2 (periodic addition)
Combined effect	Inoculated SNB + GA and H_2O_2 (periodic addition)

[a] Nutrient broth rehydrated in seawater.
[b] Glucose oxidase 1 U/ml / Catalase 0.033 U/ml.
[c] Gluconic acid.

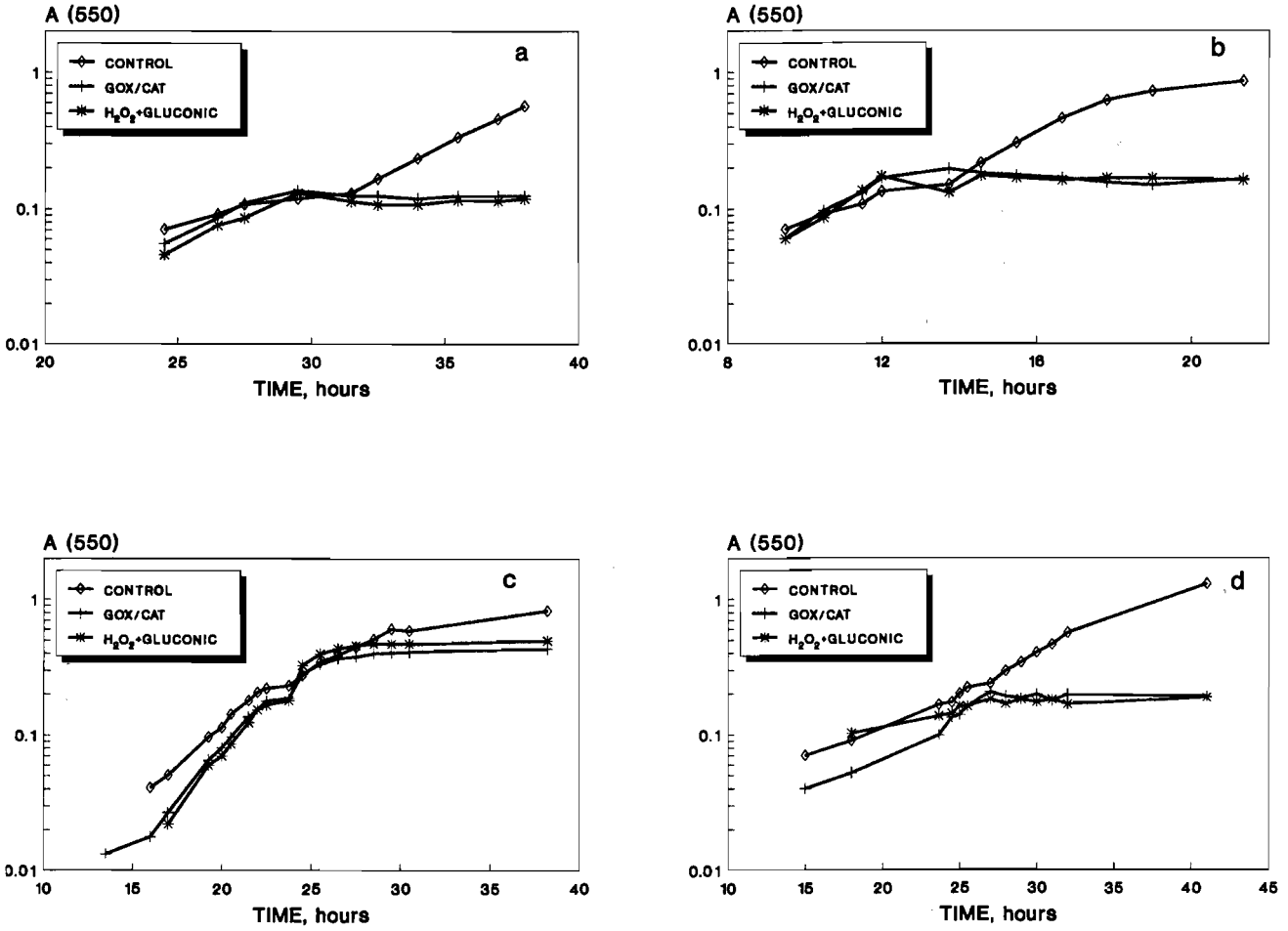

Figure 1. *Effect of simultaneous addition of hydrogen peroxide and gluconic acid on the growth of test microorganisms (a)* P. fluorescens, *(b)* A. calcoaceticum, *(c)* C. aquaticum, *(d)* H. polymorpha.

The gluconic acid versus pH curve was constructed using the following procedure. A solution with 4% glucose and 1 U/ml GOX was prepared as described above. These particular concentrations had been reported to be effective (*22*). Samples were kept at 24°C and shaken at 175 rpm in a rotary shaker. At 1-h intervals, 1-ml aliquots were removed, and pH measured with a microprocessor pH/millivolt meter (Model 811; Orion Research, Inc., Cambridge, MA) with a combination spear-tip electrode (Model 91-63; Orion Research, Inc.). Samples were diluted 1:100 with distilled water and filtered through a 0.20-μm membrane filter (Bio-Rad Laboratories, Richmond, CA). Glucose and gluconic acid were quantified by high-pressure liquid chromatography following the procedure described by Bouzas et al. (*4*). Under the chromatographic conditions used, glucose and gluconic acid coeluted. Gluconic acid concentration was determined from the UV signal where glucose does not absorb. A corrected glucose signal (refractive index detector) was determined by subtracting the calculated gluconic acid signal from the total signal.

Statistical analysis

The growth rate of the microorganisms under the different treatments was calculated by linear regression of LOG (A_{550}) versus time and compared using multiple regression analysis (*17*).

RESULTS AND DISCUSSION

Effect of gluconic acid and hydrogen peroxide

All test microorganisms were equally inhibited by glucose oxidase and by the simultaneous addition of hydrogen peroxide and gluconic acid (Fig. 1), indicating that the enzyme effect on microbial growth can be attributed to hydrogen peroxide, gluconic acid, or their combined effect. The sole addition of hydrogen peroxide at a concentration of 0.1 mM inhibited the growth of *P. fluorescens*, *H. polymorpha*, and *A. calcoaceticum* but had no effect on *C. aquaticum* (Fig. 2). The differential effect of hydrogen peroxide on these four microorganisms cannot be explained by their ability to eliminate this toxic compound for they are all catalase positive (*7*). It has been reported that microorganisms with carotenoid pigments are more resistant to hydrogen peroxide (*5*). This could be the case of *C. aquaticum* which formed yellow colonies when plated on PCA agar.

The microorganisms inhibited by hydrogen peroxide, *P. fluorescens*, *A. calcoaceticum*, and *H. polymorpha* had growth rate similar to those of the controls when gluconic acid was added to SNB (Fig. 3). This suggests that the pH reduction caused by the action of glucose oxidase had no significant effect on their growth. On the other hand, *C. aquaticum* was inhibited by the addition of gluconic acid once the pH dropped below 4.5 showing a final absorbance of 0.5 versus 0.85 for the control. The growth pattern was similar to that of the glucose oxidase treated samples, indicating that for this microorganism gluconic acid was responsible for the effect of glucose oxidase.

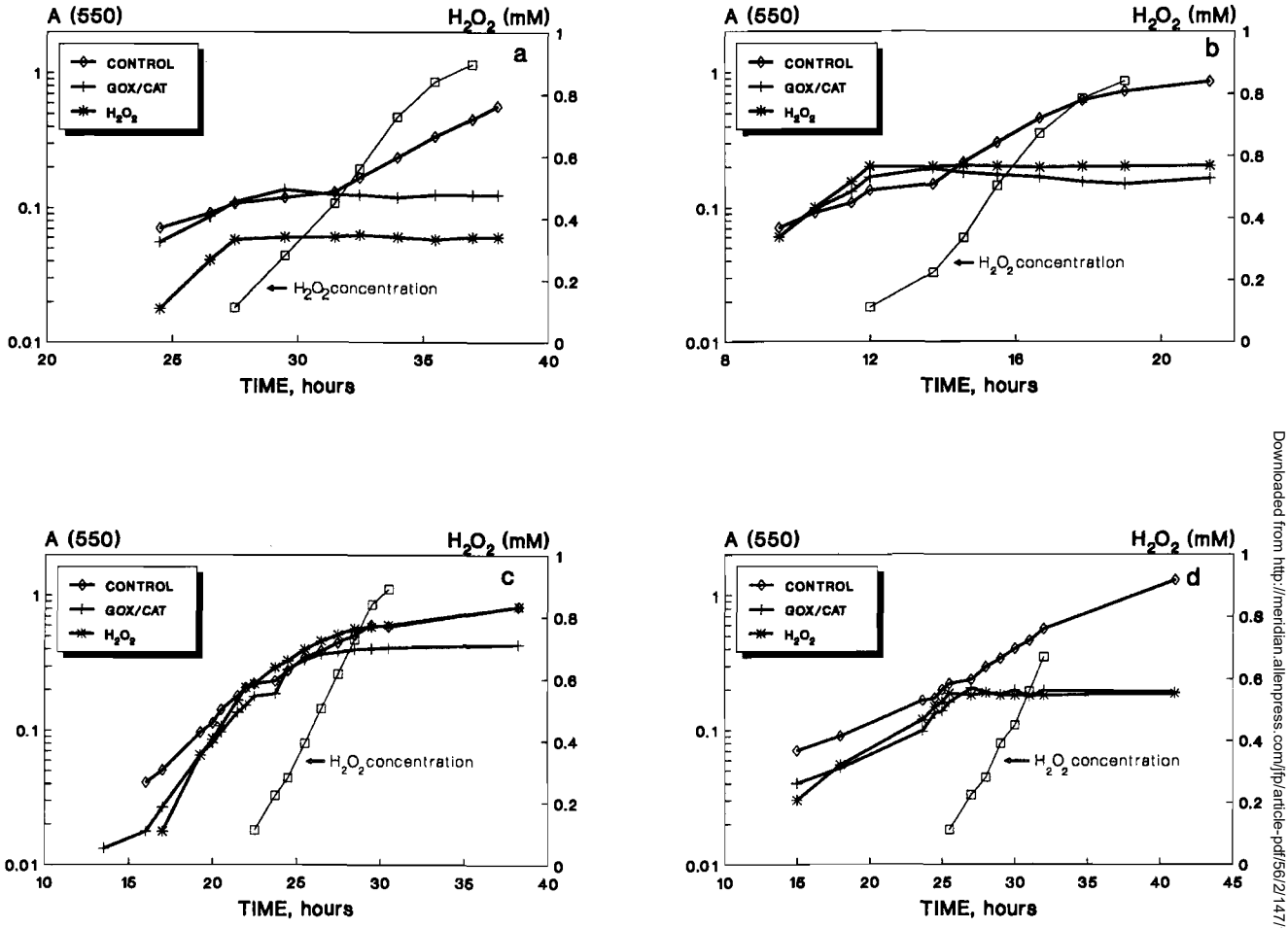

Figure 2. *Effect of hydrogen peroxide on the growth of test microorganisms (a)* P. fluorescens, *(b)* A. calcoaceticum, *(c)* C. aquaticum, *(d)* H. polymorpha.

The resistance of *P. fluorescens* strains to low pH has been reported, and at 20°C they can grow at pH as low as 4 (*6*). Vanderzant et al. (*25*) found that in beef treated with citric and lactic acids, pH 5.2 to 5.6, *Pseudomonas* spp. became the predominant species in competition with *Micrococcus* spp., *B. thermosphacta*, and *Flavobacterium* spp. Yeasts include some of the most acid-tolerant microbes found in foods, grow well below pH 4, and have a minimum growth pH around 2 (*24*).

Effect of GOX/CAT ratio

No growth was observed for 5 d at all GOX/CAT ratios when the enzymes were added to SNB immediately after inoculation (10^4 CFU/ml) with any of the four microorganisms. The growth of *P. fluorescens* treated during the exponential phase slowed significantly and then almost stopped regardless of the GOX/CAT ratio (Fig. 4a). Growth rates were calculated during the exponential phase for the control, and for the enzyme treated samples once a linear rate of absorbance change was observed after the GOX and CAT addition. Fig. 4b,c,d show similar behavior for the other test microorganisms. Growth rates for the controls and all enzyme treatments are reported in Table 3. No treatment differences were found for *P. fluorescens*, *A. calcoaceticum,* and *C. aquaticum*. In the case of *H. polymorpha*, different growth rates were detected between treatments but with no recognizable pattern.

TABLE 3. *Microbial growth rate at 24°C in nutrient broth/phosphate/seawater with different GOX/CAT ratios.*

Treatment	Growth rate h^{-1}	SE
P. fluorescens		
Control	0.313	0.011
GOX/CAT 35:1	0.0035	0.0005
GOX/CAT 5:1	0.0071	0.0007
GOX/CAT 1:1	0.0023	0.0005
A. calcoaceticum		
Control	0.334	0.018
GOX/CAT 35:1	0.003	0.0014
GOX/CAT 5:1	0.0051	0.0009
GOX/CAT 1:1	0.0097	0.0016
C. aquaticum		
Control	0.223	0.016
GOX/CAT 35:1	-0.0012	0.0005
GOX/CAT 5:1	-0.0007	0.0005
GOX/CAT 1:1	0.0007	0.0012
H. polymorpha		
Control	0.362	0.023
GOX/CAT 35:1	0.0058	0.0002
GOX/CAT 5:1	0.0090	0.0009
GOX/CAT 1:1	0.0030	0.0002

Hydrogen peroxide was shown to be the compound responsible for growth inhibition in three of the four tested

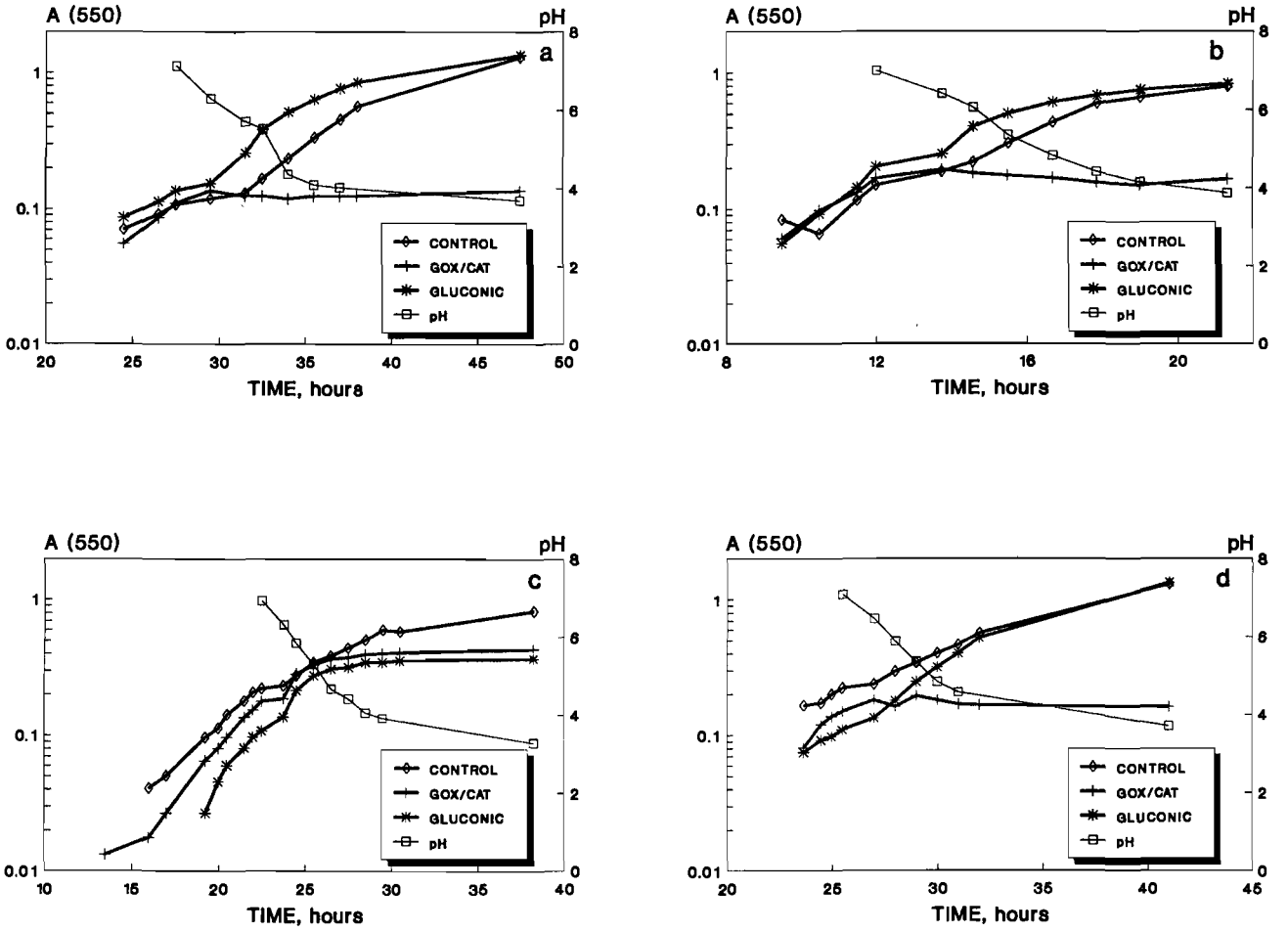

Figure 3. *Effect of gluconic acid on the growth of test microorganisms (a)* P. fluorescens, *(b)* A. calcoaceticum, *(c)* C. aquaticum, *(d)* H. polymorpha.

microorganisms (Fig. 2). Therefore, increasing amounts of catalase should reduce the effectiveness of glucose oxidase. However, the hydrogen peroxide concentration needed for growth inhibition is only 0.1 mM, and at this concentration it would decompose very slowly (*21*). The actual ratio of hydrogen peroxide production rate by GOX to its decomposition by catalase is then higher than the activity ratios (35:1, 5:1, and 1:1) would indicate.

CONCLUSIONS

In this model system, glucose oxidase was effective in inhibiting the growth of four microorganisms usually found in shrimp. The catalase level at a constant GOX concentration had no effect on the effectiveness of the enzymatic system. Hydrogen peroxide was found to cause growth inhibition of *P. fluorescens*, *A. calcoaceticum*, and *H. polymorpha* while *C. aquaticum* was inhibited by gluconic acid. Although only glucose oxidase from *Aspergillus niger* was assayed in this study, the results suggest that regardless of the degree of catalase contamination, all commercial enzyme preparations would be equally effective in microbial growth inhibition applications.

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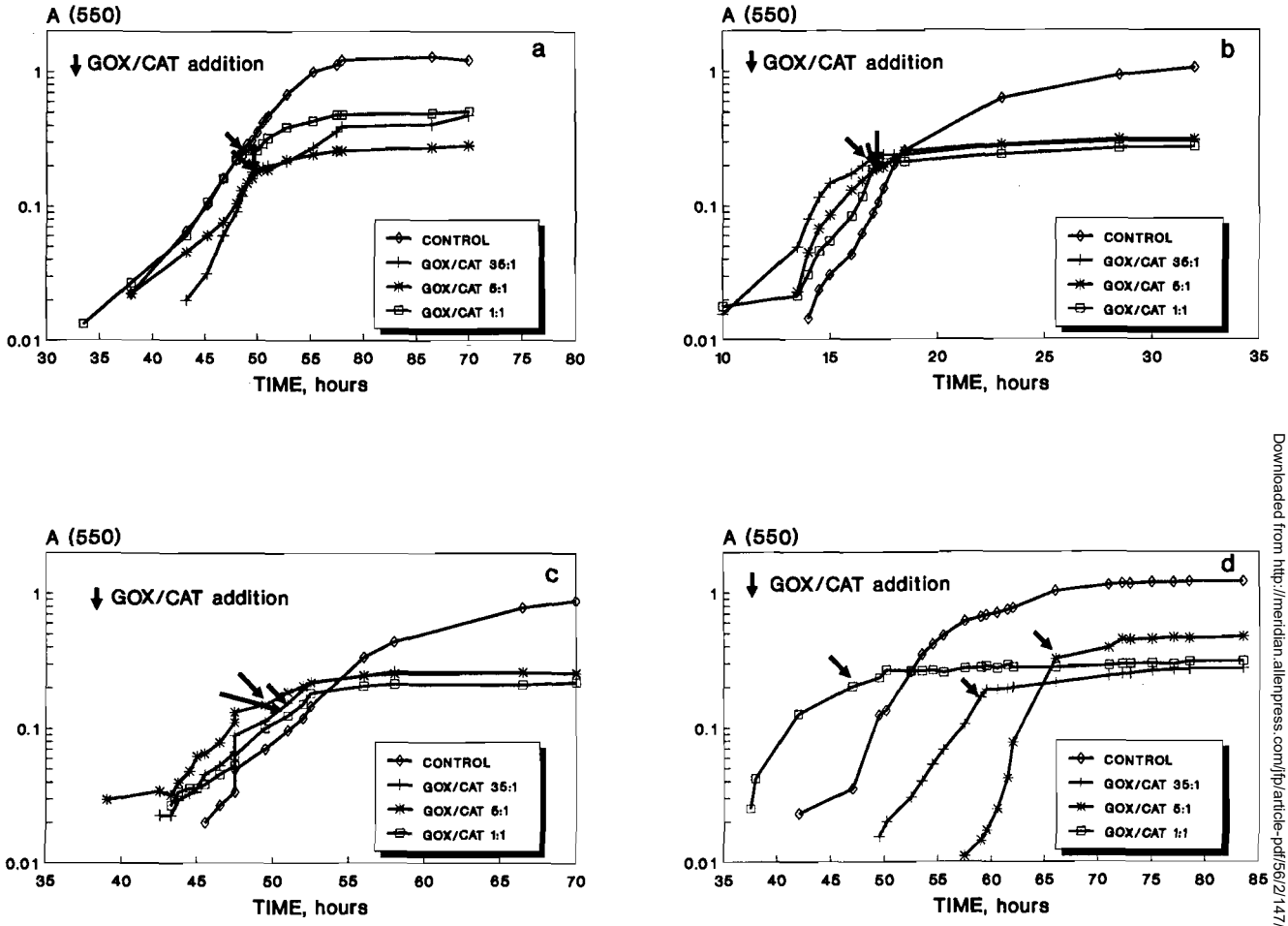

Figure 4. *Effect of different enzyme ratio on the growth of test microorganisms (a)* P. fluorescens, *(b)* A. calcoaceticum, *(c)* C. aquaticum, *(d)* H. polymorpha.

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