Effect of the Nature and Relative Concentration of Substrate, Water Mineralization, and Storage Temperature on the Oxidants Produced by Lactoperoxidase and on Their Antifungal Activity against *Penicillium expansum* and *Botrytis cinerea*

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Featured Application: The potential applications of the lactoperoxidase system are in human health and in plant pest control. However, practical applications of this natural preservative system are still limited due to various factors that can dramatically reduce its antimicrobial activity. For an industrial application, it is fundamental to understand factors affecting lactoperoxisase activity. This study is a contribution to the increased knowledge on this issue.

Abstract: Lactoperoxidase is an enzyme that generates oxidants with antimicrobial activity in presence of a (pseudo)halogen and hydrogen peroxide, but various factors can drastically reduce the antimicrobial activity of the lactoperoxidase system. Spectroscopic, ionic chromatography, and ^{13}C-NMR methods showed that the oxidants generated by lactoperoxidase are $OSCN^-$ in the presence of SCN^- and I_2 in the presence of I^-. Neither of them, however, inhibited *Penicillium expansum*, one of the causal agents of fruit mold. When a mixture of SCN^- and I^- was used, no $OSCN^-$, OCN^-, I_2, or interhalogen I_2SCN^- was produced. However, its long-term stability, NH_2-oxidizing capacity, and antifungal activity against *P. expansum* argue in favor of an I^--derived oxidant. Strongly mineralized water optimized enzyme-catalyzed reactions with higher oxidant production. Storage at 4 °C resulted in long-term stability and extended antifungal activity against *P. expansum*. The relative iodide/thiocyanate concentrations turned out to be important, as better in vitro inhibition of *Botrytis cinerea*, the causal agent of apples's grey mold, was obtained with a high KI + KSCN concentration, a KI/KSCN ratio of 4.5, and a $(KI + KSCN)/H_2O_2$ ratio of 1. The nature of the substrates, their relative concentrations, the medium, and the storage temperature modifed the antifungal activity of lactoperoxidase.

Keywords: lactoperoxidase; oxidant; antifungal activity; *Botrytis cinerea*; *Penicillium expansum*

1. Introduction

Plant fungal diseases are commonly controlled by applying chemical synthetic fungicides during farming or in packing houses. Alternative pest control methods are perceived as more environment-friendly and consumer-oriented, and should respond more adequately to the expectations of sustainable agriculture. However, compared to the number of active ingredients included in conventional chemical pesticides worldwide, the number of alternative methods currently licensed for disease control remains low.

Botrytis cinerea and *Penicillium expansum* are ubiquitous plant-pathogenic fungi whose conidia are mainly airborne, but also dispersed by water, animals, and humans [1]. *B. cinerea* can develop at cold storage temperatures and the disease spreads through rotted fruit contact [1]. *P. expansum* causes one of the most common post-harvest rots of apple [1]. Its significance has increased in recent years because it produces patulin, a mycotoxin found in rotted fruit that increases fungal virulence [2,3] and is the major cause of patulin contamination of fruit juices, compotes, and other apple-processed products [4]. In addition to Pomaceae, *P. expansum* is a saprophyte of other crops such as citrus fruit and cherries; patulin has been detected in bananas, peaches, apricots, grapes, grape juice, and wine. In Europe, patulin has been detected only in apple samples such as compotes and cooked fruit (1 µg/kg), fresh alcoholic beverages (0.12 µg/kg) and fruit (0.04 µg/kg) [4].

Lactoperoxidase (E.C.1.11.1.7; LPO) is an enzyme that occurs naturally in bovine milk and is closely related to human haem-peroxidase found in exocrine secretions such as saliva, tears, airway mucosa, or intestinal secretions. In bovine milk, LPO exerts its antibacterial activity through oxidants produced in the presence of cofactors, i.e., hydrogen peroxide (H_2O_2) and the thiocyanate ion (SCN^-) present at variable levels depending on what the cows are fed, and the iodide ion (I^-) found at very low levels in bovine milk [5–8]. The oxidants derived from SCN^- and I^- are hypothiocyanite ($OSCN^-$) ions and hypoiodite (OI^-) ions/iodine (I_2), respectively [9,10]. These oxidative products have inhibitory properties against pathogenic bacteria, fungi, and viruses [8,11–13]. Many in vitro or in vivo applications of the LPO system have been tested. This includes the preservation of raw and pasteurized milk, storage of cosmetics, and oral care products like moisturizing gel and toothpaste. Furthermore, veterinary products, preservation of foodstuffs, or plant protection have also been tested [11,14–17]. However, practical applications of the LPO system are limited. As the catalytic reactions are complex, various factors can dramatically reduce antimicrobial activity, and the transfer from in vitro to in vivo conditions has led to inconclusive data [12,18–21]. Apart from the substrates of the LPO system, the presence of other compounds in the environment of the protein may also significantly influence its efficiency. The objective of this study is to investigate the factors that affect overall enzyme efficiency to provide information necessary for the establishment of optimal conditions for the functioning of the biological activity of the LPO system and help industrials and farmers in using this natural preservation system.

2. Materials and Methods

2.1. Chemicals and Reagents

Potassium iodide, potassium thiocyanate, hydrogen peroxide 35%, 5-thio-nitrobenzoic acid, sodium sulfate, sodium cyanate, sodium sulfate, sodium chloride, sodium nitrate, and starch were purchased from VWR International. DL-Dithiothreitol and 3.3′, 5.5′-tetramethylbenzidine were purchased from Sigma-Aldrich. KS^{13}CN was obtained from Eurisotop SAS. Bentonil® was purchased from Clariant. Lactoperoxidase was purchased from Taradon Laboratory.

2.2. Oxidant Production

Lactoperoxidase mixed with clay was placed in a tea bag at a concentration of 50 units/mL and added to 1 L of water containing 150 µL of coagulant (Pac Sachtoklar, Brenntag N.V.). The mixture was mixed thoroughly for 1 min. Freshly prepared 1 M solutions of KI and/or KSCN were then added

under gentle shaking to obtain 5.4 mM KI and/or 1.2 mM KSCN (full dose), 10.8 mM KI and/or 2.4 mM KSCN (double-dose), and 2.7 mM KI and/or 0.6 mM KSCN (half-dose). Then, H_2O_2 was added in eight increments to obtain total concentrations of (pseudo)halogen and/or halogen. The solution was kept under shaking for 1 min. The tea bag containing lactoperoxidase was finally removed from the solution. Other relative concentrations of substrates were tested.

2.3. Water Mineral Characteristics

The mineral content and pH of the waters used in the study are described in Table 1. The mineral characteristics of the waters were evaluated from the dry residues, i.e., the percentages of mineral elements collected after evaporation of 1 L of water subjected to a temperature of 180 °C (NF T90-029 August 2002, Water quality—Determination of dry residues at 105 °C and 180 °C). According to the NFT90-029 standard, if dry residues are >1500 mg/L, the water is said to be rich in minerals ("High mineralization": Contrex spring water from Nestlé Belgilux S.A., Bruxelles, Belgium). When it is between 500 and 1500 mg/L, the water is qualified as medium mineralized ("Medium Low mineralization": tap water from Gembloux from distribution area n° 1324 of Brye from the Société Wallonne de Distribution des Eaux (SWDE), Nivelles, Belgium; "Medium High mineralization": Vittel spring water from Nestlé Belgilux S.A., Bruxelles, Belgium). When it is between 50 and 500 mg/L, the water is said to be weakly mineralized ("Low mineralization": Cristaline spring water Cristal-Roc from GIE Cristaline, France).

Table 1. Minerals content (mg/L) and pH of waters (values from analytical certificate analysis of water distributors).

Minerals Content mg/L	Low Mineralization Water	Medium Low Mineralization Water	Medium High Mineralization Water	High Mineralization Water
pH	Cristaline Spring: Cristal-Roc	Tap Water	Vittel	Contrex
Ca^{++}	73	131	240	468
Mg^{++}	2	18	42	74.5
Na^+	4.5	32	5	9.4
K^+	1.3	2.1	1.9	2.8
HCO_3^-	200	366	384	372
SO_4^-	20	84	400	1121
NO_3^-	<1	40	4.4	2.9
Cl^-	10	36	8	7.6
Dry residue	223	550	1084	2078
pH	7.7	7.4	7.6	7.4

2.4. Penicillium Expansum and Botrytis Cinerea Cultures

Penicillium expansum (CBS 484.75) and *Botrytis cinerea* strain V (isolated from decayed fruit in our laboratory) were grown on a potato dextrose agar at 25 °C in a culture chamber. After 10 days (*P. expansum*) or three weeks (*B. cinerea*) of incubation, conidia were collected by adding 9 mL of sterile 0.05% Tween water on the plate. Conidia were scraped off with a sterile inoculating loop and filtered through a sterilized double-layer fine cloth. The conidia concentration was adjusted using a Burker counting chamber.

2.5. Oxidants Colorimetric Dosage

Absorbances were recorded with an UV-visible spectrophotometer SmartSpec™ 3000 from Bio-Rad Laboratories. The dosage of oxidants derived from iodide can be measured alternatively through (a) the method described in [9], which is the oxidation of SH groups of the yellow TNB (5-thio-nitrobenzoic acid). This oxidation is measured at 412 nm and causes discoloration of TNB that is proportional to the oxidant concentration [9]. Furthermore, (b) the method described in details in [22], which is based on the oxidation of amine (NH_2) groups of the colorless TMB

(3.3$'$,5.5$'$-tetramethylbenzidine). This oxidation is measured at 655 nm and causes blue coloration of oxidized-TMB that is proportional to the oxidant concentration [22].

The dosage of oxidants derived from thiocyanate can only be measured with the oxidation of SH-group of TNB ([9]), as their oxidation state is not sufficient to oxidize NH_2 groups.

Iodine titration was measured by sodium thiosulfate titration [23].

2.6. Ion Chromatography

The determination of soluble anions was carried out using a DX-120 ion chromatograph (Dionex, Sunnyvale, CA, USA). The column was an Ion Pac AS9-HC and the pre-column an Ion Pac AG9-HC. The eluent was 9 mM Na_2CO_3/$NaHCO_3$, pH 12. The flow-rate was set to $1.0\,\text{mL}\,\text{min}^{-1}$. All ions were detected by conductivity. Lactoperoxidase catalytic reaction were performed in MilliQ$^®$ water (Merck Chemicals S.A.) with the addition of (a) hydrogenocarbonate (584 mg/L Mg(HCO_3)$_2$) under CO_2-bubbling, stirring, and warming (60 $°$C) until the solution was colorless and (b) chloride (5 mg/L Cl^-) and nitrate (5 mg/L $NO_3{}^-$).

Pure potassium iodide, potassium thiocyanate, sodium cyanate, sodium sulfate, sodium chloride, and sodium nitrate were injected to assess retention times. The retention time of $OSCN^-$ was measured with a chemically prepared solution of $OSCN^-$, according to the method described in [9,24]. Briefly, the lead thiocyanate Pb(SCN)$_2$ was prepared; the precipitate was filtered and washed with ice water and dried in the dark under vacuum on $CaCl_2$. Lead thiocynanate was diluted in CCl_4 to obtain 0.27 mM. 1 mL of 0.14 mM Br_2 were added in aliquots and vortexed until the Br_2 solution becomes colorless. The excess lead salt was removed by centrifugation and decantation. The solution of (SCN)$_2$ was used the same day of its preparation and conserved in the dark at 4 $°$C. The concentration of (SCN)$_2$ was measured at 296 nm assuming a molar extinction coefficient of 140 $M^{-1}\,cm^{-1}$. Finally, 1 mL of 9 mM (SCN)$_2$ was added, drop by drop, to 10 mL of 0.1 M cold NaOH and vigorously stirred using a vortex. The emulsion was separated into two phases by centrifugation at 4 $°$C for 10 min at 2500 rpm and the $OSCN^-$ fraction was measured by oxidation of SH group at 283 µM and 285 µM (experiment 1), then at 323 µM and 321 µM (experiment 2). Overall, two independent production of chemical $OSCN^-$ were performed.

2.7. ^{13}C Nuclear Magnetic Resonance Measurements

^{13}C spectra were recorded at 20 $°$C on a Bruker AVIII HD 400 MHz apparatus using a 5-mm broad-band probe (SmartProbeTM). Samples were placed in an 8-inch thin-walled NMR sample tube. Using direct carbon detection with proton decoupling during relaxation delay and acquisition, one-dimensional spectra were collected. The following parameters were used: a spectral window of 239 ppm, 64 k points, an acquisition time of 1.363 s, a recycle delay of 2 s; 2048 scans were added. Prior to Fourier-transform, the free induction decay was multiplied by a negative exponential with a line broadening of 1Hz. Chemical shifts were referenced relatively to DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) from Sigma. NMR samples were prepared with 540-µL samples, 60 µL of deuterium oxide, and 2 µL of DSS 60 mM. Samples were analyzed within 12 h to 48 h after preparation and kept at 4 $°$C in the dark before NMR analysis. The relative intensities of the signals were analyzed and integrated using MestReNova software (version 10).

2.8. In Vitro Antifungal Tests

The pathogens at a final concentration of 1.10^6 spores/mL were cultured for five days in threefold-diluted sterile potatodextrose broth in the presence or absence (control) of an inhibiting solution, in the dark at 22 $°$C in ELISA plate wells, following the protocol of [25]. The controls were prepared in four replicates. The inhibiting ion solutions were prepared in eight replicates. Optical densities (ODs) at 650 nm were measured after 48 h and 120 h of incubation and mean ODs at 120 h

were calculated to perform data analysis. The percentage of inhibition of a pathogen was calculated using the following formula [25]:

$$\% \text{ inhibition} = (\text{OD}_{\text{control}} - \text{OD}_{\text{inhibiting solution}})/(\text{OD}_{\text{control}}) \times 100$$

where $\text{OD}_{\text{control}}$ is the optical density of the pathogen culture after subtracting the optical density of the medium, and $\text{OD}_{\text{inhibiting solution}}$ is the optical density of the inhibiting solution-treated pathogen culture after subtracting the optical density of the medium and of the inhibiting solution.

The tests were performed in two independent assays. Data were analyzed using MINITAB version 17. A one-way parametric analysis of variance (ANOVA) was performed on optical density mean values. When the analysis was statistically significant ($p < 0.001$), the means were separated using Tukey's multiple comparison test. Differences at $p < 0.05$ were considered as significant.

The effect of substrate alone (KI, KSCN and H_2O_2) on the growth of *P. expansum* and *B. cinerea* have been performed in the reference [11,25] and no inhibitory effect of substrates alone have been observed.

3. Results

3.1. Influence of the Nature of the Substrates on the Oxidants and on Their Antifungal Activity

After the LPO oxidoreduction of 1.2 mM KSCN + 5.4 mM KI + 6.6 mM H_2O_2, 5.4 mM KI + 5.4 mM H_2O_2, and 1.2 mM KSCN + 1.2 mM H_2O_2, as well as the removal of the enzyme, the three solutions were kept at 25 °C. The oxidation of thiol groups and the biological activity against *P. expansum* was measured over 10 days. Results are presented in Table 2. No inhibition was observed with the oxidant solutions prepared from SCN^- or I^-. The oxidant solutions were prepared with a mixture of SCN^- and I^- inhibited fungal growth by 80 to 94% for 10 days. Catalytic oxidation of iodide produced stable low levels of oxidant for up to 10 days, with a yellowing after enzymatic oxidation. Iodine was titrated with sodium thiosulfate at 0.092 mM. Catalytic oxidation of thiocyanate produced high levels of oxidant on the production day and then a sharp loss after 1 day of storage, with a half-life of less than 1 day. Ion chromatography revealed the presence of SCN^-, SO_4^{2-}, OCN^-, and $OSCN^-$ (Figure 1).

Table 2. In vitro inhibition % of *P. expansum* and $-SH$ oxidant concentration (µM) with various doses of substrates after oxidoreduction by lactoperoxidase and storage at 25 °C for 0, 1, 2, 3, 5, or 10 days. Tukey's comparison of means was applied with 95% confidence; means that do not share a letter are significantly different; ns, not significantly different from the control. Means and s.d. of two independent experiments.

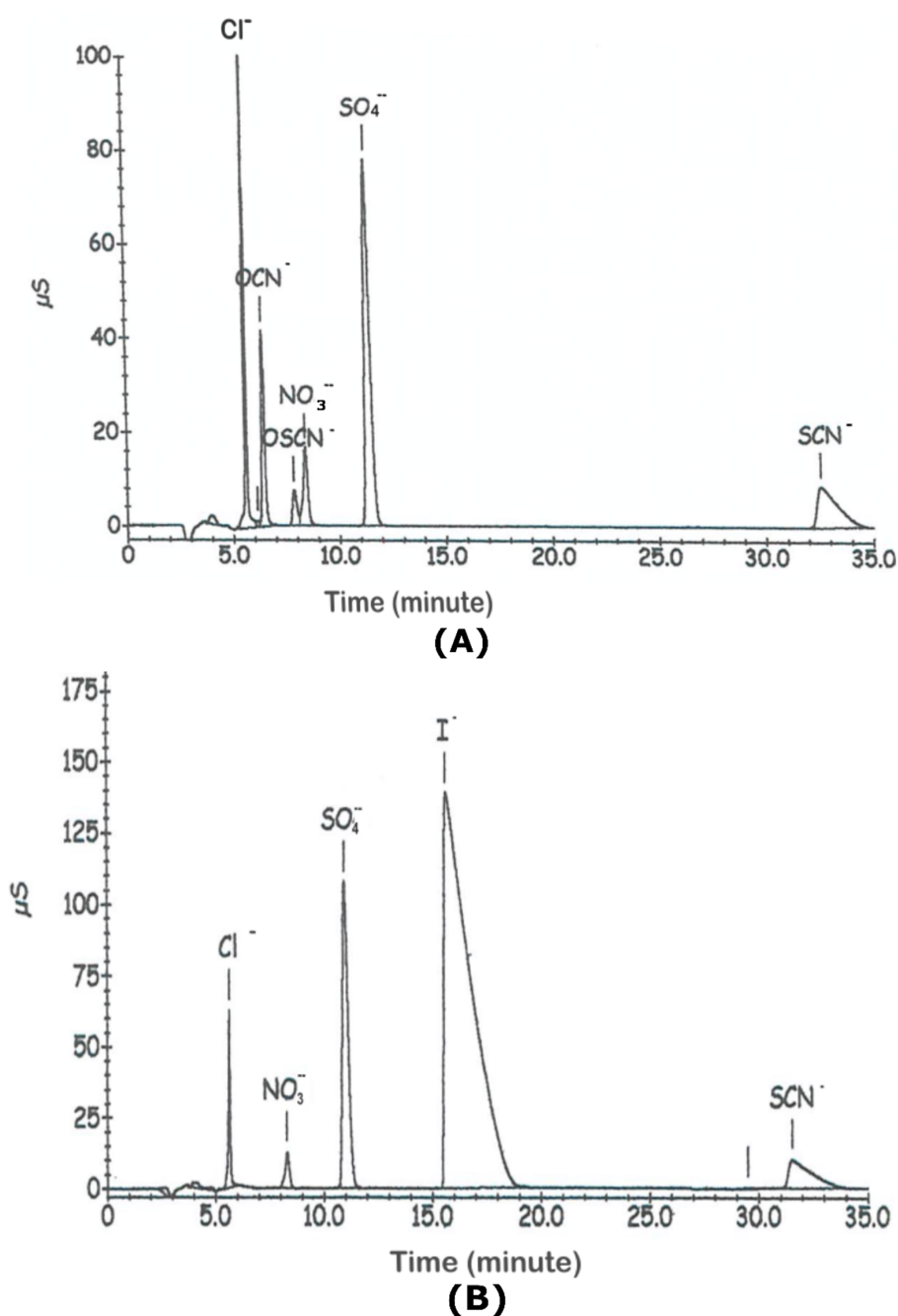|	**Substrate Doses**		
	1.2 mM KSCN + 1.2 mM H_2O_2	**1.2 mM KSCN + 5.4 mM KI + 6.6 mM H_2O_2**	**5.4 mM KI + 5.4 mM H_2O_2**
Storage (days)	***P. expansum* inhibition (%)**		
0	0 ± 4 [ns]	94 ± 3 [a]	0 ± 1 [ns]
1	-	94 ± 8 [a]	-
2	-	90 ± 3 [a]	-
3	-	86 ± 3 [a]	-
5	-	94 ± 5 [a]	-
10	-	80 ± 5 [a]	-
	Oxidant Concentration (µM)		
0	359 ± 24	405 ± 80	54 ± 6
1	72 ± 9	381 ± 28	55 ± 4
2	17 ± 7	361 ± 34	52 ± 4
3	10 ± 5	362 ± 28	45 ± 6
5	0 ± 0	277 ± 50	49 ± 11
10	0 ± 0	263 ± 28	52 ± 9

Figure 1. Ion chromatography of (**A**) 1.2 mM KSCN + 1.2 mM H$_2$O$_2$ and (**B**) of 1.2 mM KSCN + 5.4 mM KI + 6.6 mM H$_2$O$_2$ after oxidoreduction by lactoperoxidase in Milli-Q$^®$ water. Representative spectra of two independent experiments is given.

Catalytic oxidation of the iodide-thiocyanate mixture produced a colorless solution containing stable and highly dosed oxidant with a shelf-life of more than 10 days (Table 2). Iodine titration was performed; no iodine was measured. Ion chromatography of the SCN$^-$- and I$^-$-derived solution detected I$^-$, SCN$^-$, and SO$_4^{2-}$, but did not detect any OSCN$^-$, OCN$^-$, or OI$^-$ (Figure 1).

^{13}C NMR was performed on two solutions: 1.2 mM KS^{13}CN + 1.2 mM H$_2$O$_2$ and 1.2 mM KS^{13}CN + 5.4 mM KI + 6.6 mM H$_2$O$_2$ (Figure 2). It confirmed the presence of OCN$^-$ and OSCN$^-$ in the SCN$^-$-oxidized solution [26] and the absence of OSCN$^-$, OCN$^-$ or interpseudohalogen molecules, such as ICN, I(SCN)$_2^-$, and I$_2$SCN$^-$ in the SCN$^-$- and I$^-$-oxidized solutions [25].
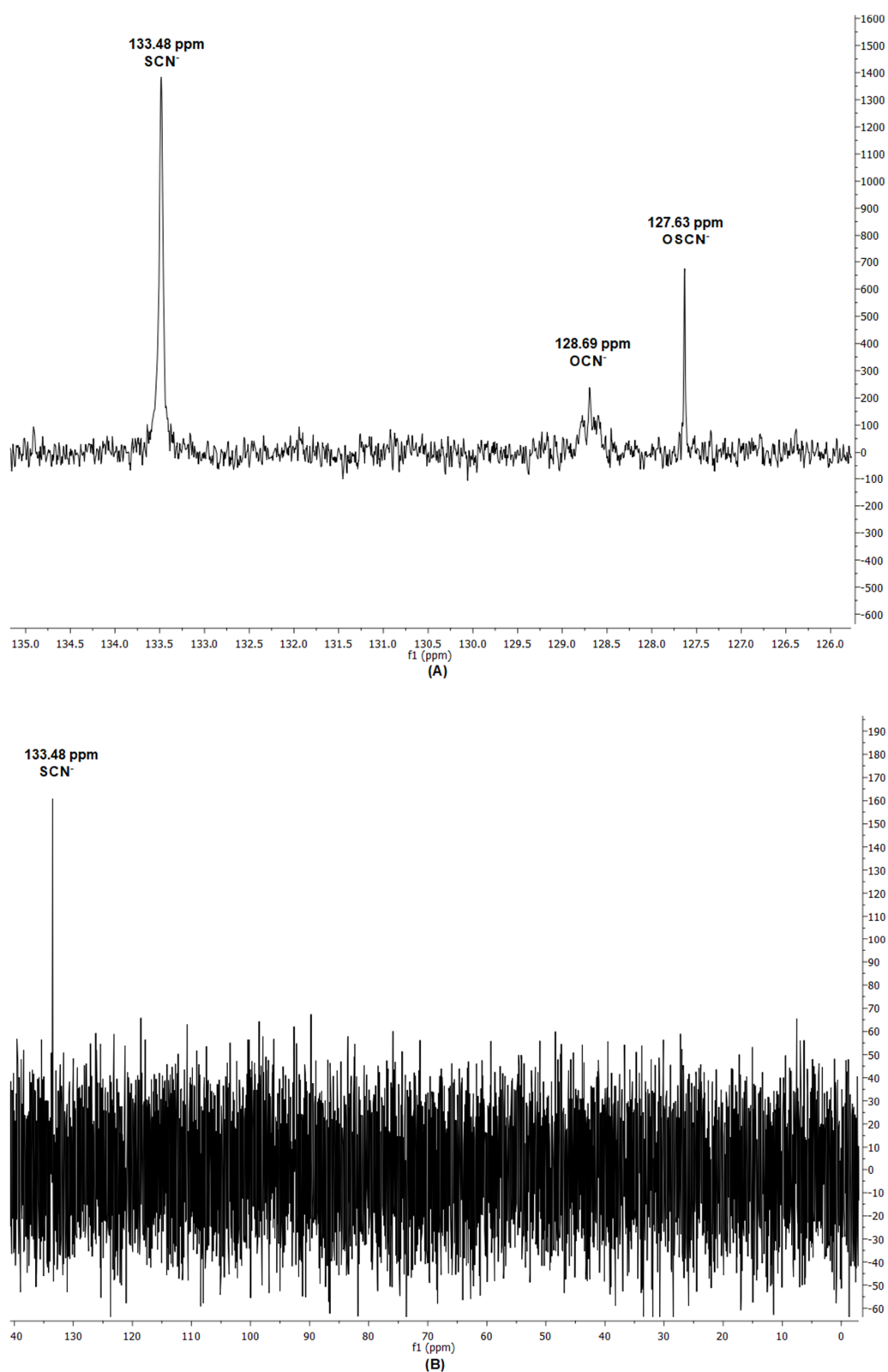
Figure 2. ^{13}C NMR chemical shift of (**A**) 1.2 mM KS^{13}CN + 1.2 mM H$_2$O$_2$ or (**B**) 1.2 mM KS^{13}CN + 5.4 mM KI + 6.6 mM H$_2$O$_2$ after lactoperoxidase oxidoreduction in tap water. Representative spectra of two independent experiments are given.

3.2. Influence of Water Mineralization on Oxidant Production

We used four waters with various mineralization levels (low, medium low, medium high, or high) to carry out the oxidoreduction of 1.2 mM KSCN + 5.4 mM KI + 6.6 mM H_2O_2 (full dose), 0.6 mM KSCN + 2.7 mM KI + 3.3 mM H_2O_2 (half dose), and 2.4 mM KSCN + 10.8 mM KI + 13.2 mM H_2O_2 (double dose) by 50 U/mL of LPO (Table 3). In the full dose, oxidant production was favored by water mineralization, i.e., lower mineralization gave the lowest oxidant production and higher mineralization gave the highest oxidant concentration. The effect of mineralization on oxidant production was less pronounced with half-doses of substrates. Conversely, it was more pronounced with double-dosed substrates: high mineralization induced a 1.78-fold increase of oxidant production in regards to the lowest mineralized water.

Table 3. Oxidant concentration measured as $-NH_2$ oxidation (μM) after LPO oxidoreduction of 2.7 mM KI + 0.6 mM KSCN + 3.3 mM H_2O_2 (half-dose); 5.4 mM KI + 1.2 mM KSCN + 6.6 mM H_2O_2 (full dose) and 10.8 mM KI + 2.4 mM KSCN + 13.2 mM H_2O_2 (double-dose) in low- to high-mineralized waters. Means and s.d. of two independent experiments are given.

	Water Mineralization			
	Low	**Medium Low**	**Medium High**	**High**
Substrate Doses	**Oxidant Concentration (μM)**			
Half-dose	291 ± 20	254 ± 44	235 ± 32	256 ± 16
Full-dose	383 ± 25	407 ± 17	503 ± 33	538 ± 42
Double-dose	546 ± 24	563 ± 18	785 ± 29	974 ± 49

3.3. Influence of the Storage Temperature on Oxidant Stability and Antifungal Activity

The influence of the storage temperature is showed in Figure 3. Storage at 4 °C noticeably increased the shelf-life of the oxidant, whatever the substrate dose and the initial oxidant concentration. The solutions prepared with double-dose substrate concentrations contained higher initial oxidant doses, but also underwent a higher degradation rate. Full-dose solutions prepared with high mineralized waters (Medium High and High) and double-dose solutions prepared with low mineralized waters (Low and Medium low) showed similar initial oxidant doses of around 500 μM. However, the shelf-life at 25 °C was around 90 days for the full dose and 25 days for the double dose. At 4 °C, it was more than 360 days for the full dose and 120 days for the double dose.

The biological activity of the oxidant was clearly related to its dose (Tables 4–6), which implies that the rate of oxidant degradation affects biological activity duration. The remanence of biological activity was influenced by the storage temperature and the initial oxidant dose. Cold storage of the oxidant and reduced amounts of substrates (half and full doses) prolonged the biological activity of the oxidant. The storage temperature also seemed to affect the minimum inhibitory dose, with enhanced efficiency of the oxidants kept at 4 °C. At 25 °C, the oxidant dose necessary for biological activity was around 205 μM for the half dose, 157 μM for the full dose, and 219 μM for the double dose. At 4 °C, the oxidant dose necessary for biological activity was around 150 μM for the half dose, 111 μM for the full dose, and 172 μM for the double dose.
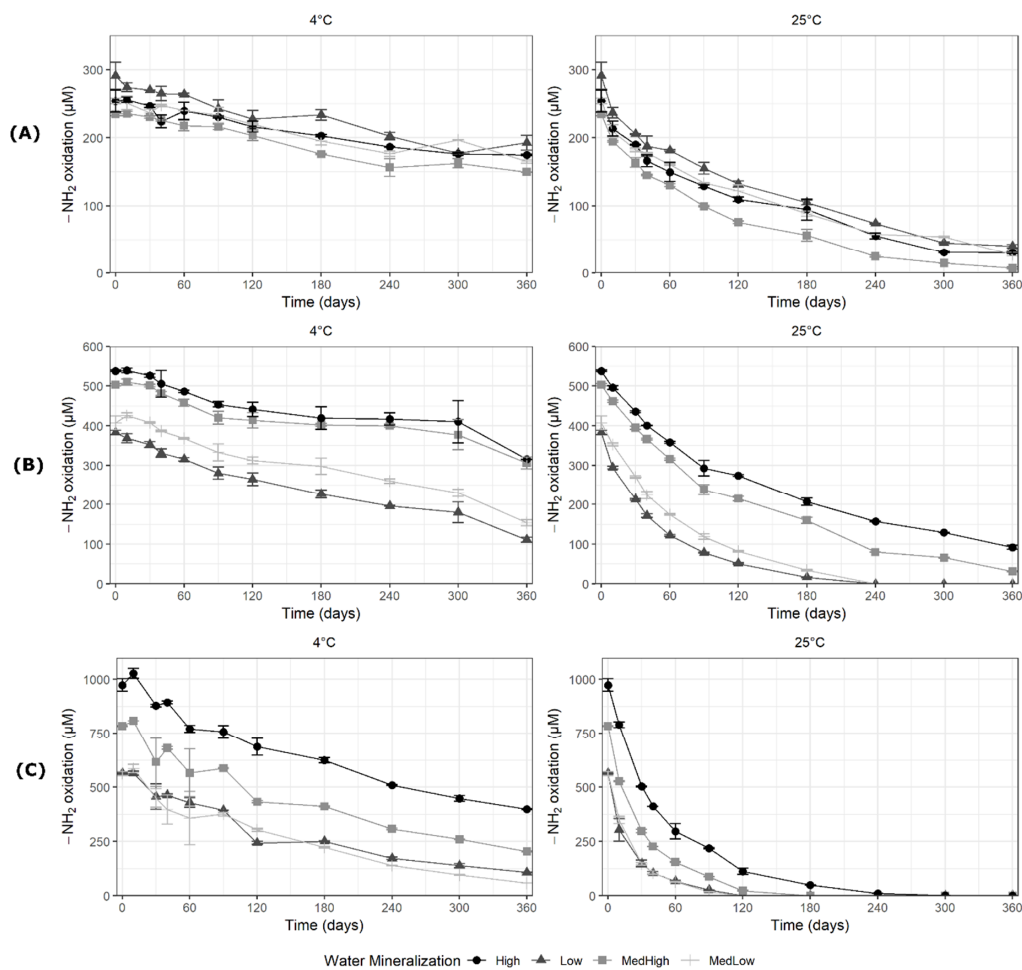
Figure 3. Oxidant concentration expressed in $-NH_2$ oxidation (μM) of (**A**) (half-dose): 2.7 mM KI + 0.6 mM KSCN + 3.3 mM H_2O_2; (**B**) (full-dose): of 5.4 mM KI + 1.2 mM KSCN + 6.6 mM H_2O_2; (**C**) (double-dose): 10.8 mM KI + 2.4 mM KSCN + 13.2 mM H_2O_2 in differentially mineralized (low, medium low, medium high, and high) waters after lactoperoxidase oxidation and over 360 days of storage at 4 °C or 25 °C. Means and s.d. of two independent experiments are given. MedLow: Medium low; MedHigh: Medium High.

Table 4. Percentage of inhibition of *P. expansum* growth by half-dose solutions (2.7 mM KI + 0.6 mM KSCN + 3.3 mM H_2O_2) after lactoperoxidase oxidoreduction in low- to high-mineralized waters and storage at 25 °C or 4 °C for 360 days. Tukey's comparison of means was applied with 95% confidence; means that do not share a letter are significantly different; ns, not significantly different from the control. Temp.: storage temperature. Means and s.d. of the two independent experiments are given.

		Storage Duration (Days)				
		0	**30**	**90**	**240**	**360**
Temp.	**Water Mineralization**	*P. expansum* **Inhibition (%)**				
	Low	91 ± 5 [b]	81 ± 4 [a]	26 ± 4 [c]	0 ± 6 [ns]	4 ± 2 [ns]
25 °C	**Medium Low**	88 ± 7 [b]	24 ± 10 [ns]	0 ± 2 [ns]	0 ± 2 [ns]	0 ± 4 [ns]
	Medium High	92 ± 2 [b]	0 ± 1 [ns]	5 ± 5 [ns]	0 ± 4 [ns]	7 ± 7 [ns]
	High	89 ± 3 [b]	0 ± 0 [ns]	0 ± 1 [ns]	3 ± 6 [ns]	0 ± 5 [ns]
	Low	100 ± 0 [a]	73 ± 8 [a]	95 ± 3 [a]	95 ± 3 [a]	$95 \pm$ [a]
4 °C	**Medium Low**	99 ± 1 [a]	89 ± 5 [a]	91 ± 2 [a]	96 ± 4 [a]	$92 \pm$ [a]
	Medium High	100 ± 0 [a]	73 ± 7 [a]	53 ± 3 [bc]	$61 \pm$ [c]	$90 \pm$ [a]
	High	100 ± 0 [a]	75 ± 4 [a]	63 ± 11 [b]	$85 \pm$ [b]	$90 \pm$ [a]

Table 5. Percentage of inhibition of *P. expansum* growth by full-dose solutions (5.4 mM KI + 1.2 mM KSCN + 6.6 mM H_2O_2) after lactoperoxidase oxidoreduction in low- to high-mineralized waters and storage at 25 °C or 4 °C for 360 days. Tukey's comparison of means was applied with 95% confidence; means that do not share a letter are significantly different; ns, not significantly different from the control. Temp.: storage temperature. Water mineral.: water mineralization. Means and s.d. of two independent experiments are given.

		Storage Duration (Days)						
		0	**30**	**90**	**150**	**240**	**300**	**360**
Temp.	**Water Mineral.**	*P. expansum* Inhibition (%)						
	Low	89 ± 2 [b]	98 ± 1 [ab]	10 ± 7 [ns]	14 ± 10 [ns]	17 ± 11 [ns]	6 ± 5 [ns]	2 ± 1 [ns]
25 °C	**Medium Low**	89 ± 1 [b]	96 ± 2 [ab]	8 ± 5 [ns]	3 ± 2 [ns]	15 ± 8 [ns]	2 ± 1 [ns]	4 ± 3 [ns]
	Medium High	92 ± 0 [ab]	95 ± 4 [ab]	90 ± 4 [a]	85 ± 4 [a]	2 ± 1 [ns]	0 ± 0 [ns]	5 ± 4 [ns]
	High	95 ± 0 [a]	91 ± 5 [a]	93 ± 5 [a]	86 ± 5 [ab]	89 ± 6 [a]	1 ± 1 [ns]	1 ± 1 [ns]
	Low	90 ± 5 [ab]	97 ± 2 [ab]	100 ± 0 [a]	98 ± 3 [b]	92 ± 5 [a]	93 ± 4 [a]	94 ± 5 [a]
4 °C	**Medium Low**	90 ± 1 [ab]	99 ± 1 [b]	92 ± 3 [a]	89 ± 5 [ab]	89 ± 5 [a]	92 ± 1 [a]	92 ± 6 [a]
	Medium High	91 ± 3 [ab]	93 ± 3 [ab]	87 ± 0 [a]	83 ± 5 [a]	89 ± 4 [a]	92 ± 0 [a]	91 ± 4 [a]
	High	94 ± 1 [ab]	94 ± 3 [ab]	88 ± 2 [a]	85 ± 6 [a]	90 ± 6 [a]	92 ± 3 [a]	92 ± 5 [a]

Table 6. Percentage of inhibition of *P. expansum* growth by double-dose solutions (10.8 mM KI + 2.4 mM KSCN + 13.2 mM H_2O_2) after lactoperoxidase oxidoreduction in low- to high-mineralized waters and storage at 25 °C or 4 °C for 360 days. Tukey's comparison of means was applied with 95% confidence; means that do not share a letter are significantly different; ns, not significantly different from the control. Temp.: storage temperature; Water mineral.: water mineralization; M. Low: medium low; M. High: medium high. Means and s.d. of two independent experiments are given.

		Storage Duration (Days)						
		0	**30**	**90**	**120**	**180**	**240**	**300**	**360**
Temp.	**Water Mineral.**	*P. expansum* Inhibition (%)						
	Low	94 ± 3 [a]	14 ± 10 [ns]	0 ± 0 [ns]	10 ± 4 [ns]	0 ± 1 [ns]	0 ± 0 [ns]	2 ± 1 [ns]	0 ± 0 [ns]
25 °C	**M. Low**	93 ± 4 [a]	0 ± 0 [ns]	0 ± 0 [ns]	8 ± 2 [ns]	6 ± 2 [ns]	2 ± 1 [ns]	5 ± 3 [ns]	3 ± 2 [ns]
	M. High	91 ± 2 [a]	88 ± 8 [a]	0 ± 0 [ns]	4 ± 4 [ns]	5 ± 3 [ns]	9 ± 4 [ns]	4 ± 2 [ns]	2 ± 1 [ns]
	High	91 ± 4 [a]	87 ± 5 [a]	88 ± 1 [a]	0 ± 0 [ns]	0 ± 0 [ns]	3 ± 3 [ns]	0 ± 1 [ns]	0 ± 0 [ns]
	Low	99 ± 1 [b]	88 ± 4 [a]	90 ± 0 [a]	91 ± 1 [a]	88 ± 3 [a]	94 ± 1 [a]	31 ± 10 [a]	59 ± 5 [a]
4 °C	**M. Low**	98 ± 2 [b]	89 ± 4 [a]	89 ± 3 [a]	91 ± 2 [a]	87 ± 2 [a]	6 ± 3 [ns]	1 ± 1 [ns]	10 ± 3 [ns]
	M. High	99 ± 0 [b]	86 ± 3 [a]	88 ± 3 [a]	92 ± 2 [a]	85 ± 4 [a]	92 ± 2 [a]	90 ± 5 [b]	89 ± 4 [b]
	High	98 ± 2 [b]	90 ± 6 [a]	88 ± 4 [a]	90 ± 3 [a]	84 ± 3 [a]	91 ± 0 [a]	97 ± 4 [b]	92 ± 5 [b]

3.4. Influence of the Relative Substrate Concentrations on Oxidant Levels and Antifungal Activity

3.4.1. Influence of the Relative Substrate Concentrations on Oxidant Levels

We classified the relative iodide/thiocyanate concentrations in three categories: <1 (Figure 4), =1 (Figure 5) and >1 (Figure 6), and measured the influence of each ratio through the level of produced oxidant. When thiocyanate is the major substrate present in the media (KI/KSCN ratio < 1; Figure 4), oxidant production stayed low unless thiocyanate was added at a high concentration (10 mM KSCN). The capacity to oxidize the $-NH_2$ group remained low, with a maximum concentration at 25 µM, even with a high iodide concentration. As the NH_2-dosage was very low and clearly different from SH-dosage, it can be hypothesized that SH-dosage is predominantly due to oxidized thiocyanate. However, even with 10 mM KSCN, the SH-dosage reached only 250 µM, which is low when compared with 350 µM $OSCN^-$ produced with 1.2 mM KSCN as the sole substrate (Table 2). The addition of iodide act like an inhibitor of $OSCN^-$ production. Three ratios showed this clearly: (i) 1.2 mM KI + 5.4 mM KSCN, (ii) 1.2 mM KI + 4.05 mM KSCN, and (iii) 1.2 mM KI + 1.35 mM KSCN, which represent 4.5, 3.37, and 1.12, respectively, more thiocyanate than iodide, as well as where $OSCN^-$ production increased with thiocyanate quantity and NH_2-dosage increased with iodide concentration relatively to thiocyanate. The lactoperoxidase system that used a mixture of iodide and thiocyanate in

which thiocyanate is the major substrate represent a less-effective way of oxidants production and a substrate-expensive way of use of the LPO-system.
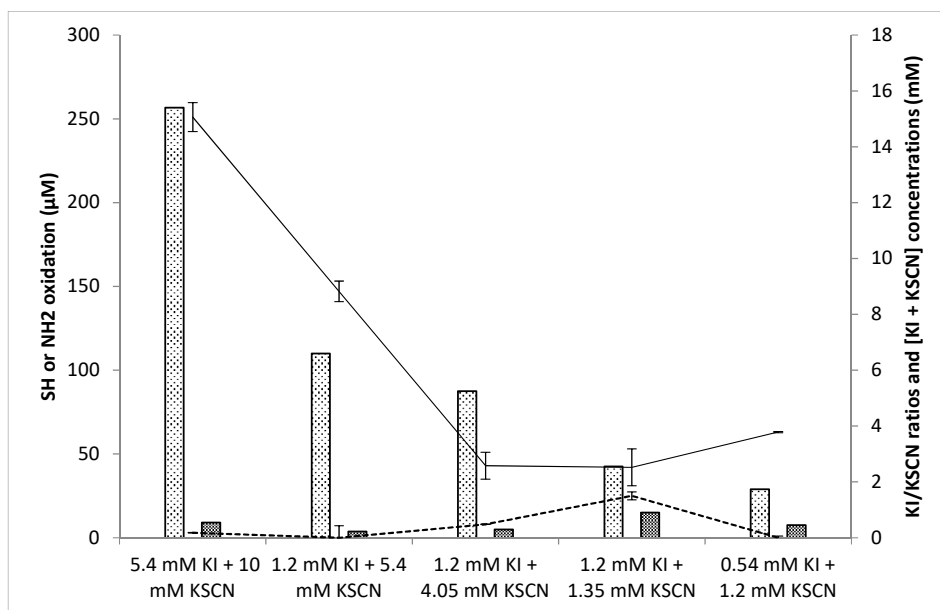

Figure 4. KI/KSCN ratio < 1. Oxidation levels of −SH (plain line) and −NH$_2$ (dashed line) (µM), total pseudo-halogen concentrations (light-colored bars) (mM), and KI/KSCN ratios (dark-colored bars) (mM) of various doses of (KI + KSCN) after lactoperoxidase oxidoreduction in tap water. Means and s.d. of the two independent experiments are given.
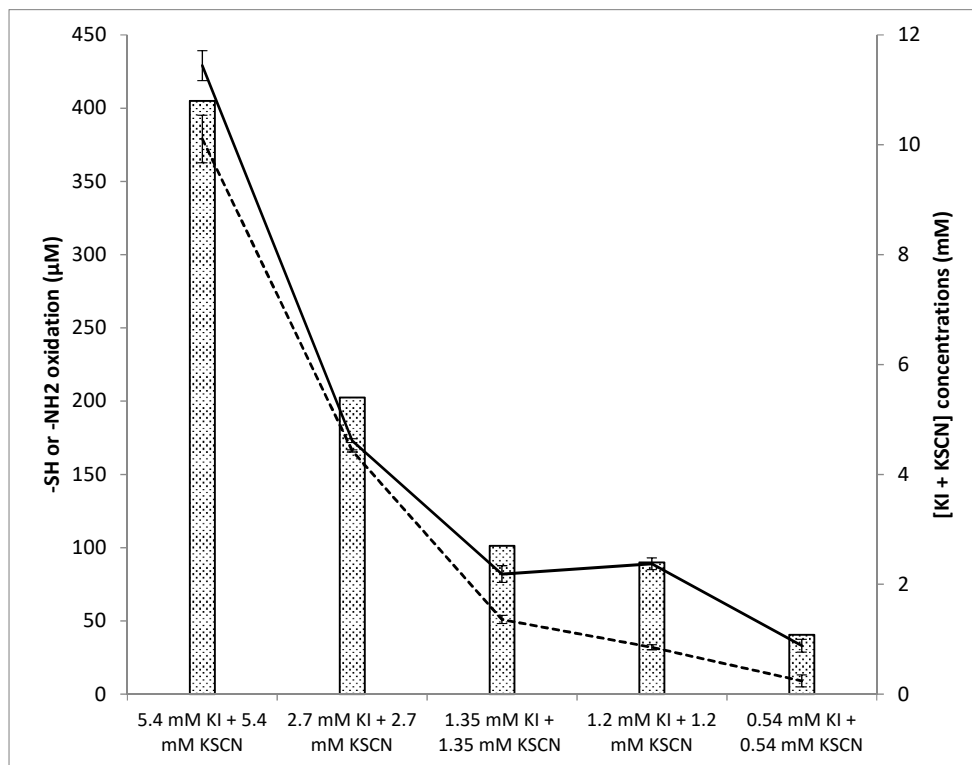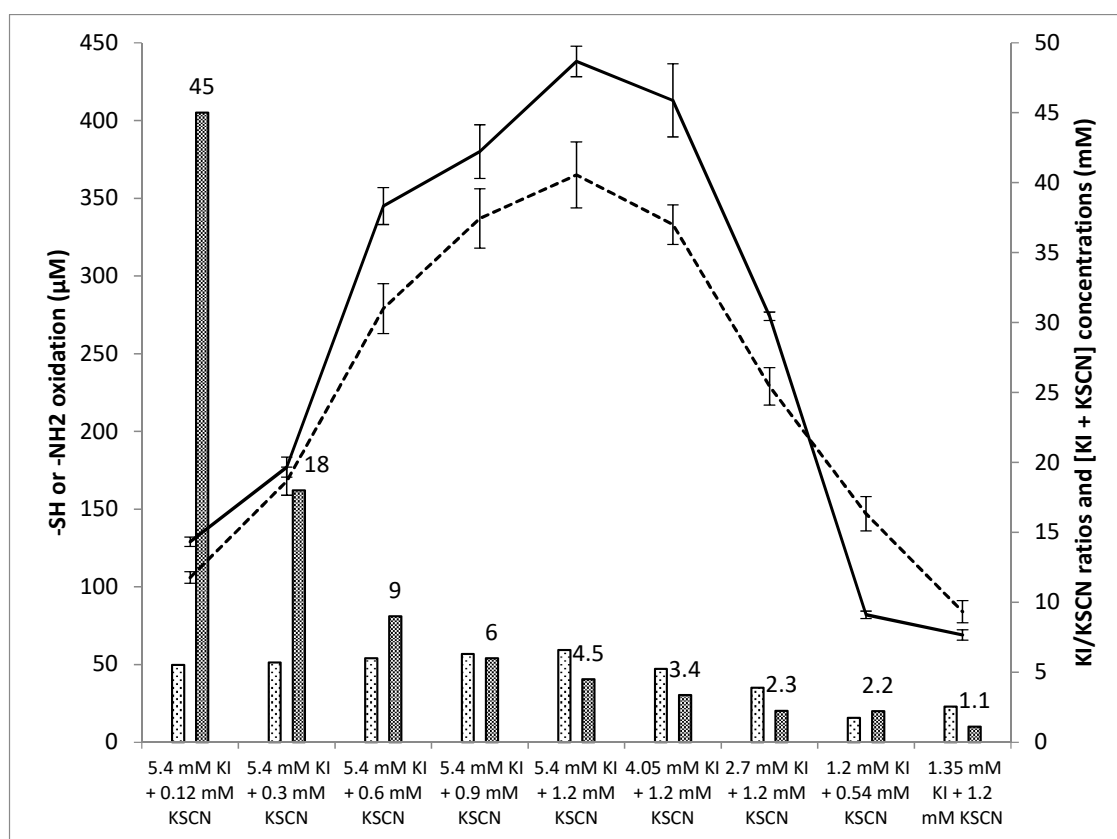

Figure 5. KI/KSCN ratio = 1. Oxidation levels of −SH (plain line) and −NH$_2$ (dashed line) (µM), and total pseudo-halogen concentrations (light bars) (mM) of various doses of (KI + KSCN) after lactoperoxidase oxidoreduction in tap water. Means and s.d. of the two independent experiments are given.

Figure 6. KI/KSCN ratio > 1. Oxidation levels of −SH (plain line) and −NH$_2$ (dashed line) (μM), total pseudo-halogen concentrations (light-colored bars) (mM), and KI/KSCN ratios (dark-colored bars) (mM) of various doses of (KI + KSCN) after lactoperoxidase oxidoreduction in tap water. Means and s.d. of the two independent experiments are given.

When the same amount of thiocyanate and iodide are added in the media (KI/KSCN ratio = 1; Figure 5), the enzyme is able to produced high doses of oxidants if the total amount of substrate added are high. The SH-dosage is relatively close to the NH$_2$-dosage, so that it can be hypothesized that oxidant are mainly represented by iodide-oxidized oxidant. The oxidation of iodide seems to be more effective when both substrates increased. However, the use of equal amount of iodide and thiocyanate are less-effective in oxidants production, especially with regards to the total quantity of substrates added (10.8 mM) that reached about 400 μM oxidant when compared with total substrate of 6.6 mM (5.4 mM KI + 1.2 mM KSCN) and the same amount of 400 μM oxidants produced (Table 2).

When more iodide is added relative to thiocyanate (KI/KSCN ratio > 1; Figure 6), the oxidant production curve was bell-shaped, with an optimal ratio around 4.5, and a less favorable ratio above 9 or below 2.5. If the ratio moved closer to 1, the oxidant production decreased, as if the ratio increased above 9. A ratio above 9 means that thiocyanate dropped, and could mean that oxidation of a precise level of thiocyanate is essential for an optimal level of iodide-derived oxidant production.

3.4.2. Influence of the Substrate Concentrations on Antifungal Activity

We measured the influence of the relative substrate concentrations on *B. cinerea* inhibition (Table 7). We tested 14 KI/KSCN/H$_2$O$_2$ ratios at 3 dilutions each. The oxidant induced better inhibition with (i) high KI + KSCN concentrations, (ii) a KI/KSCN ratio of 4.5, and (iii) a (KI + KSCN)/H$_2$O$_2$ ratio of 1. Increased dilution combined with lower (KI + KSCN) concentrations reduced biological efficiency.

When the KI/KSCN ration was close to 1 and the total (pseudo)halogen concentration was low, the capacity of the oxidant to inhibit the fungus was lost. This was also observed with low H$_2$O$_2$ doses.

Table 7. Total [KI + KSCN] dose (mM), KI/KSCN ratio, [KI + KSCN + H_2O_2] doses (mM), dilution factor, percentage of inhibition of *B. cinerea* growth by various doses of (KI + KSCN +H_2O_2) after lactoperoxidase oxidoreduction. The doses were grouped to observe the influence of the total pseudohalogen and halogen concentrations, of the KI/KSCN ratio, and of the H_2O_2 concentration. Means and s.d. of the two independent trials are given.

[KI + KSCN] (mM)	KI/KSCN Ratio	[KI-KSCN-H_2O_2] (mM)	Dilution Factor	*B. cinerea* Inhibition (%)
Influence of the total [KI + KSCN] concentration				
6.6 mM	4.5	5.4-1.2-6.6	1:3	88 ± 5
		5.4-1.2-6.6	1:5	84 ± 4
		5.4-1.2-6.6	1:10	83 ± 2
4.4 mM	4.5	3.6-0.8-3.6	1:3	88 ± 5
		3.6-0.8-3.6	1:5	88 ± 6
		3.6-0.8-3.6	1:10	70 ± 11
3.3 mM	4.5	2.7-0.6-3.3	1:3	88 ± 6
		2.7-0.6-3.3	1:5	83 ± 10
		2.7-0.6-3.3	1:10	34 ± 1
5.6 mM	7.84	5-0.64-5.64	1:3	92 ± 0
		5-0.64-5.64	1:5	69 ± 11
		5-0.64-5.64	1:10	42 ± 6
3.95 mM	7.84	3.5-0.45-3.95	1:3	71 ± 11
		3.5-0.45-3.95	1:5	42 ± 5
		3.5-0.45-3.95	1:10	32 ± 8
2.25 mM	7.84	2-0.25-2.25	1:3	38 ± 0
		2-0.25-2.25	1:5	39 ± 6
		2-0.25-2.25	1:10	29 ± 7
Influence of the KI/KSCN ratio				
1.12 mM	2.3	0.78-0.34-1.2	1:3	90 ± 4
		0.78-0.34-1.2	1:5	25 ± 0
		0.78-0.34-1.2	1:10	22 ± 0
3.9 mM	2.25	2.7-1.2-3.9	1:3	84 ± 1
		2.7-1.2-3.9	1:5	81 ± 2
		2.7-1.2-3.9	1:10	24 ± 7
2.95 mM	2.25	1.35-0.6-1.9	1:3	87 ± 3
		1.35-0.6-1.9	1:5	52 ± 4
		1.35-0.6-1.9	1:10	20 ± 12
2.4 mM	1.1	1.35-1.2-2.5	1:3	28 ± 4
		1.35-1.2-2.5	1:5	31 ± 4
		1.35-1.2-2.5	1:10	26 ± 5
1.3 mM	1.1	0.67-0.6-1.27	1:3	16 ± 6
		0.67-0.6-1.27	1:5	17 ± 6
		0.67-0.6-1.27	1:10	13 ± 2
Influence of the H_2O_2 concentration				
2.64 mM	4.5	2.16-0.48-0.5	1:3	10 ± 7
		2.16-0.48-0.5	1:5	12 ± 3
		2.16-0.48-0.5	1:10	20 ± 2
2.64 mM	4.5	2.16-0.48-1.1	1:3	39 ± 3
		2.16-0.48-1.1	1:5	10 ± 9
		2.16-0.48-1.1	1:10	7 ± 7
2.64 mM	4.5	2.16-0.48-2.7	1:3	80 ± 3
		2.16-0.48-2.7	1:5	45 ± 5
		2.16-0.48-2.7	1:10	14 ± 1

4. Discussion

4.1. Nature of the Reaction Product

It is well established that haem-peroxidases oxidize SCN^- into $OSCN^-$ and I^- into HOI/I_2 in the presence of H_2O_2 [9,10]. While studying biological lactoperoxidase activity in the presence of an SCN^-/I^- mixture, a few articles have addressed the products generated under such and such

experimental conditions [11,12,21,27]; several have showed that a more stable oxidative complex was formed in specific pH and ionic strength conditions [25,27–29]. We found $OSCN^-$ after LPO oxidation of SCN^- and I_2 after LPO oxidation of I^-. $OSCN^-$ is not able to oxidize amine(NH_2)-group [8,22], because of its relatively weak oxidative strength that makes $OSCN^-$ specific regarding its molecular target, i.e., a thiol moiety. This implies a lower antimicrobial activity, particularly against fungus, as shown in our study and the reviewed literature [22,30,31]. The iodine/hypoiodite ions have a stronger oxidative strength that allows them both to oxidize thiol and amine groups [32–35]. However, its antimicrobial effect can be hampered by the catalytic reaction between iodine and H_2O_2, and could explain the low level of oxidant produced and the absence of antifungal activity in our study [36]. In iodide and thiocyanate mixtures, there is a strong competition between SCN^- and I^- to fix LPO; I^- hampers the interaction of SCN^- with LPO, inducing a 10-fold increase of the dissociation constant (K_D) of SCN^- from LPO [37]. In other words, the affinity of LPO for SCN^- decreases, so that in our experimental conditions the quantity of generated oxidants decreased, too [37]. Based on various distinct techniques, our results show that $OSCN^-$, OCN^-, and I_2 were not present as stable oxidants, whereas SO_4^{2-} was a stable byproduct. SO_4^{2-} was measured in high concentrations, demonstrating that SCN^- was oxidized. ^{13}C NMR showed that no interhalogen such as ICN, $I(SCN)_2^-$, or I_2SCN^- was observable [25,38]. Anionic chromatography did not detect OI^-. OI^- stability is relative because aqueous I_2 has a very complex behavior; consequently, OI^- stability strongly depends on matrix interactions and on the pH of the medium [8]. The chromatography technique separated each reaction product during the analytical process; this may have reduced OI^- stability [8,39–41]. On the other hand, the stability of the oxidant, when stored at 4 °C over a long period of time, its NH_2-oxidizing capacity, and its capacity to inhibit fungi, argue in favor of an I^--derived active ion [8,12,35].

4.2. Influence of Water Mineralization

Water mineralization strongly influenced on the amount of produced oxidant. Higher mineralization increased the oxidant concentration, especially with concentrated substrate doses. The ratios between the lowest oxidant level, which corresponded to low mineralization, and the highest oxidant level, which corresponded to high mineralization, in the presence of half-dose, full-dose, and double-dose substrates were 1.23, 1.4, and 1.78, respectively, demonstrating that mineralization played a tremendous role in the presence of elevated substrate levels.

The role of minerals has already been showed with ammonium sulfate, which allows horseradish and microbial peroxidases to work more effectively [42]. In particular, both stabilizing and activating effects were found with a cationic surfactant such as benzalkonium chloride [43]. Ammonium sulfate ((NH_4)$_2SO_4$) increased LPO activity, therefore positive charges are determining for enzyme activity [43]. The high-mineralized waters used in this study contained high levels of Ca^{2+} and Mg^{2+}, which could explain why more oxidants were generated. The four waters had similar pH values and the pH was unlikely to play a major role in the differences in oxidant concentrations observed among waters through a modification of the reductant potential of Compound I/native LPO [44].

The functionality of haem peroxidases is influenced by the heme Fe^{3+}/Fe^{2+} redox couple and by the protein-matrix interaction [45]. In fact, LPO is stabilized by iron-ligand binding interactions, by electrostatic interactions, by the protein environment, and by the solvent [45]. It is important to stabilize and optimize the redox functionality of lactoperoxidase when highly concentrated substrates are used, especially for H_2O_2 whose redox couple is elevated [46] and it seems that concentrated minerals in the media optimize the catalytic reaction of LPO when elevated substrate levels are used. Better stabilization of the ferric heme could increase H_2O_2 consumption and formation of LPO Compound I, which in turn could induce a higher consumption of iodide and thiocyanate and could finally generate a higher quantity of reaction products.

4.3. Influence of the Storage Temperature on the Stability and Antifungal Activity of the Oxidant

The half-lives of the half dose, full dose, and double dose in several mineralized waters after storage at 4 °C or 25 °C are summarized in Table 8. Cold storage obviously prolonged the stability of the oxidant. Higher substrate doses increased oxidant instability independently of the storage temperature. Higher instability of $OSCN^-$ in the presence of higher SCN^-, H_2O_2, and $OSCN^-$ concentrations has already been described [12]. Although the nature of the ion was different in our case, it seems to have followed the same principles.

Table 8. Half-lives ($T_{1/2}$) expressed in days of the double-, full and half-doses of substrates after lactoperoxidase oxidoreduction in various mineralized waters (low; medium low; medium high; high) in function of the storage temperature (25 °C or 4 °C).

	$T_{1/2}$ (Days)					
	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C
Water Mineralization	**Double-Dose**		**Full-Dose**		**Half-Dose**	
Low	11	34	49	309	109	494
Medium Low	15	119	36	248	100	464
Medium High	20	165	98	504	73	433
High	33	229	121	491	94	529

4.4. Influence of the Relative Substrate Concentrations

The relative thiocyanate and iodide concentrations turned out to be important. In particular, a higher I^- concentration relative to SCN^-, enough H_2O_2, and a high total amount of SCN^- and I^-, proved to be important. Enough H_2O_2 is obviously necessary to activate the native enzyme in Compound I; this step is essential to oxidize (pseudo)halogens thereafter. If the H_2O_2 dose is too low, lower oxidant doses are produced. When iodide is added in lower or similar doses to thiocyanate, iodide interfere with the amount of oxidant produced. As the amount of oxidant decreases, the inhibiting efficiency against *B. cinerea* droped. When iodide and thiocyanate are present, there is a strong competition between SCN^- and I^- to fix LPO. Therefore, when the two substrates are added to LPO in equal amounts, they most probably inhibit each other through an antagonism between I^- and SCN^- to fix the binding site of lactoperoxidase [12]. Increased oxidant quantities are produced with elevated substrates and the better inhibition observed in this case is probably due to a dose effect, as already observed with other pathogens [11].

5. Conclusions

The literature shows that the LPO-catalyzed oxidation of I^- and SCN^- mixtures can result in contradictory antimicrobial efficiency. The present article shows that certain mixtures of lactoperoxidase-oxidized I^- and SCN^- have a very good antimicrobial potential. Practical applications should use an optimal iodide/thiocyanate ratio of 4.5 to favor antimicrobial efficiency and a strongly mineralized medium if high doses of substrates are used. Although the oxidant is relatively stable, its long-term storage is clearly improved at 4 °C. The use of an environment that enhances the oxidoreduction reaction catalyzed by the enzyme can dramatically increase its efficiency; as a consequence, more oxidants are generated and antimicrobial activity is better.

Conflicts of Interest: The authors declare no conflict of interest.

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