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Antimicrobial action of palmarosa oil (*Cymbopogon martinii*) on *Saccharomyces cerevisiae*

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Abstract

The essential oil extracted from palmarosa (*Cymbopogon martinii*) has proven anti-microbial properties against cells of *Saccharomyces cerevisiae*. Low concentrations of the oil (0.1%) inhibited the growth of *S. cerevisiae* cells completely. The composition of the sample of palmarosa oil was determined as 65% geraniol and 20% geranyl acetate as confirmed by GC–FTIR. The effect of palmarosa oil in causing K^+ leakage from yeast cells was attributed mainly to geraniol. Some leakage of magnesium ions was also observed. Blocking potassium membrane channels with caesium ions before addition of palmarosa oil did not change the extent of K^+ ion leakage, which was equal to the total sequestered K^+ in the cells. Palmarosa oil led to changes in the composition of the yeast cell membrane, with more saturated and less unsaturated fatty acids in the membrane after exposure of *S. cerevisiae* cells to the oil. Some of the palmarosa oil was lost by volatilization during incubation of the oil with the yeast cells. The actual concentration of the oil components affecting the yeast cells could not therefore be accurately determined.

Keywords: *Cymbopogon martinii*; Graminaceae; Palmarosa oil; Geraniol; Geranyl acetate; *Saccharomyces cerevisiae*

1. Introduction

Essential oils of plants show anti-microbial activity against a wide range of bacteria including antibiotic resistant species and fungal species (Carson et al., 1995; Carson and Riley, 1995). They can affect both Gram-positive and Gram-negative bacteria in addition to yeasts and filamentous fungi (Delaquis et al., 2002; Hammer et al., 1996; Hili et al., 1997; Lachowicz et al., 1998; Pattnaik et al., 1995, 1996, 1997). One oil of commercial importance is palmarosa oil, its antifungal action being attributed mainly to its geraniol content (Bard et al., 1988). This oil is valued highly in the perfumery industry as a source of high-grade geraniol (Mallavarapu et al., 1998). It is fungistatic against the filamentous fungi *Aspergillus niger*, *Chaetomium globosum* and *Penicillium funiculosum* (Delespaul et al., 2000) and is considered to provide protection against mosquitoes (*Anopheles culcifacies*) (Ansari and Razdan, 1995). *Cryptococcus neoformans*, a fungus which causes infection during the last stages of AIDS is inhibited both by palmarosa oil and geraniol (Viollon and Chaumont, 1994).

The principal components of palmarosa oil are recorded as geraniol and geranyl acetate, with lesser quantities of linalool and β-caryophyllene (Mallavarapu et al., 1998), though variations in composition occur in different extractions depending on the plant material and method of oil extraction. Reports of some essential oils affecting membrane integrity inc!ude tea tree (*Melaleuca alternifolia*) oil causing damage to membranes in *Candida albicans* while other oils and their components have disrupted the permeability barrier of yeast cells (Bard et al., 1988; Cox et al., 2000).

To understand more fully the antimicrobial action, the effect of palmarosa oil and its major constituents on *Saccharomyces cerevisiae* were investigated, with respect to ion leakage from cells, changes in composition of the cell membrane and metabolism of oil components.

2. Results and discussion

The composition of the sample of palmarosa oil used in this study was ascertained to be 65% geraniol, 20%

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geranyl acetate by GC–FTIR in comparison with authentic samples (Fig. 1).

The antimicrobial effects of the oil, geraniol and geranyl acetate on *S. cerevisiae* were determined using the drop diffusion technique on MYGP plate cultures. The volume of each component of the oil used in these inhibition tests reflected their proportion in the oil, for example 5 μl of palmarosa oil, or 3.25 μl of geraniol, or 1 μl of geranyl acetate. The diameters of zones of inhibition were measured after 24 h. Eighty five percent of the inhibitory effects of the oil (25 mm zone) could be attributed to the additive action of geraniol (17.7 mm zone) and geranyl acetate (3.7 mm zone) (Fig. 2). Palmarosa oil at 0.1% concentration inhibited the growth of *S. cerevisiae* cells completely (Fig. 3).

When cells of S. cerevisiae were incubated with either palmarosa oil or geraniol, leakage of potassium ions from the cells was detected (Fig. 4). Incubation with geranyl acetate did not result in more K^+ release than from the control cells without oil. After 30 mm incubation, K^+ released by the oil was 488 μmol/1.5×10^9 cells and that released by geraniol was 528 μmol/1.5×10^9 cells, but with longer incubation up to 120 mm palmarosa oil caused release of 970 μmol/1.5×10^9 cells, whereas geraniol had no further effect. This leakage coincided with the maximum sequestered potassium ions in cells of *S. cerevisiae*, determined using Triton X-100 (973 ± 77 μmol/1.5×10^9 cells). Potassium leakage from a different fungus, *C. albicans*, due to action of geraniol over a period of 2 h has been reported earlier (Bard et al., 1988). Cox et al. (1998) also reported potassium ion leakage due to essential oils such as tea tree oil in bacteria.

Release of magnesium ions from *S. cerevisiae* was less than that of potassium ions after incubation (120 mm) with palmarosa oil (92 μmol/1.5×10^9 cells) and geraniol (31 μmoles/1.5×10^9 cells). No more Mg^{2+} ions were released as a result of incubation with geranyl acetate than from the control cells (Fig. 5). There was no leakage of Ca^{2+} due to either the oil or its major components (data not shown).

Therefore the only major oil component that contributed to the effect of the oil itself in releasing K^+ and Mg^{2+} ions from yeast cells was geraniol. For these ions to be released from the cells, some changes in the cell membrane may have occurred. The composition of the cell membrane, as determined by analysis of fatty acid methyl esters derived from membrane fatty acids of cells of *S. cerevisiae* confirmed previous reports that the major components were C16:0, C16:1, C18:0 and C18:1 (Augustyn and Kock, 1989). Table 1 shows the effect of palmarosa oil on membrane fatty acid composition, expressed as the mean percentage of each lipid (C16:0, C 16:1, C18:0, C18:1) extracted from cells after incubation with 10 μg oil per ml of culture broth. Palmarosa oil caused the proportion of C16:0 and C18:0 fatty acids (saturated) to increase, while C16:1 fatty acids (unsaturated) decreased, reflecting a change in membrane

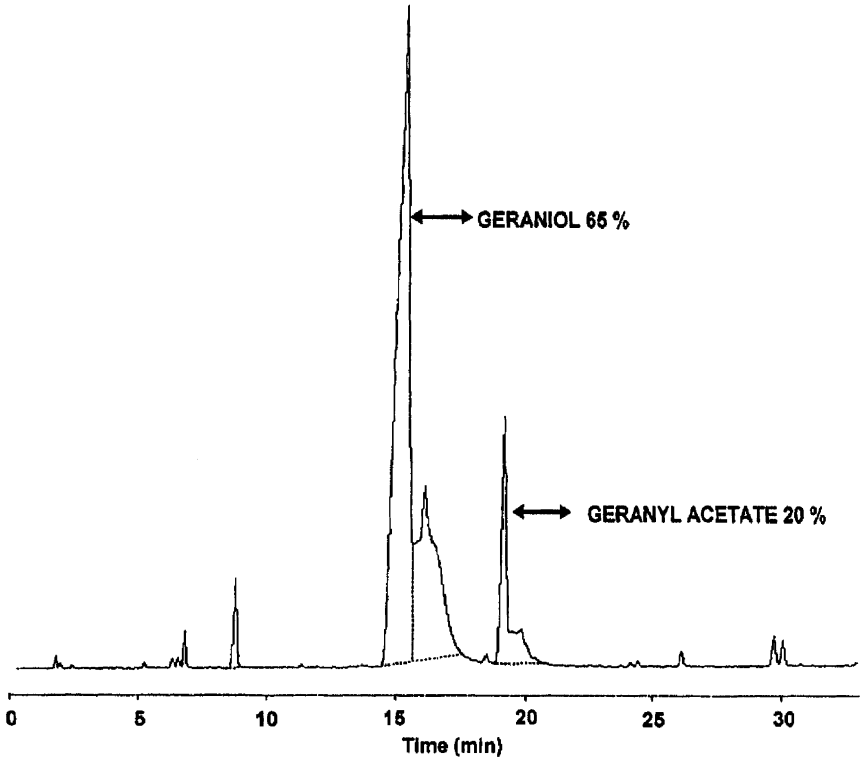

Fig. 1. Separation of components of palmarosa oil by gas chromatography on a SPB 35 column (30 m × 0.75 mm).

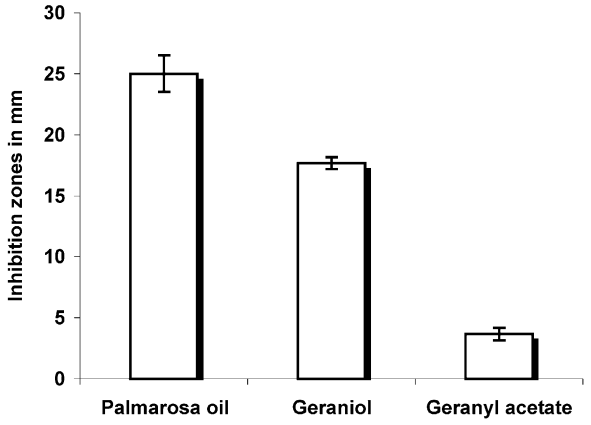

Fig. 2. Inhibition zones of growth of *S. cerevisiae* with palmarosa oil and its major components, geraniol and geranyl acetate.

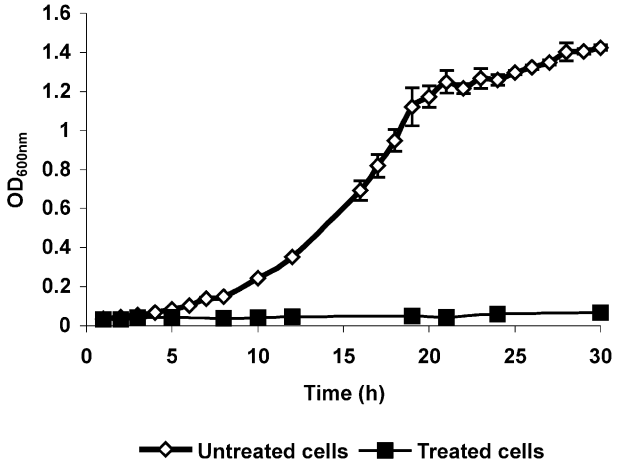

Fig. 3. Growth curve of *S. cerevisiae* with and without incubation with 0.1% palmarosa oil.

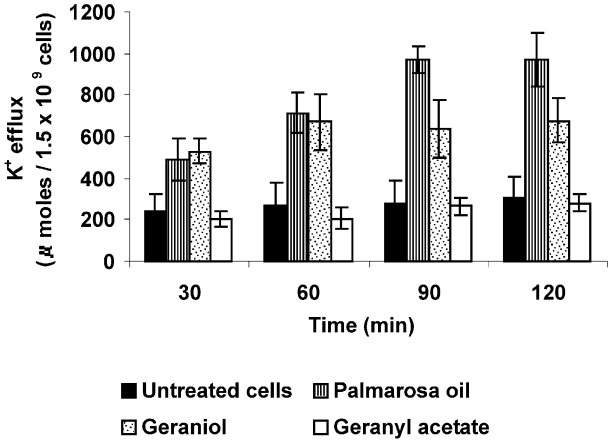

Fig. 4. Potassium leakage from cells of *S. cerevisiae* on treating with μl/ml palmarosa oil (0.1%), 0.6 μl/ml geraniol and 0.2 μl/ml geranyl acetate.

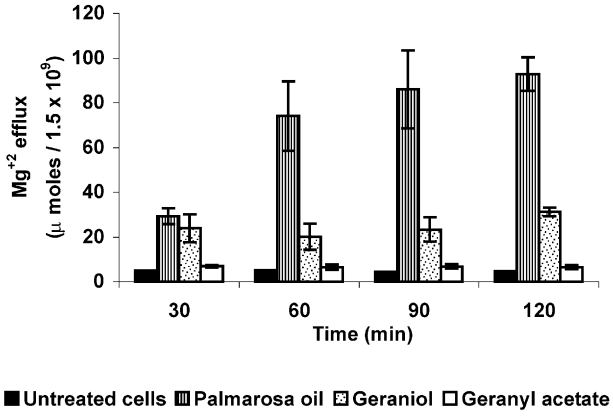

Fig. 5. Magnesium leakage from cells of *S. cerevisiae* on treating with 1 μl /ml palmarosa oil (0.1%), 0.6 μl /ml geraniol and 0.2 μl/ml geranyl acetate.

Table 1
The effect of palmarosa oil on fatty acids (derivatized to methyl esters) extracted from *S. cerevisiae*

Lipid	% of total fatty acids (S.D.)	
	Cells with no oil	Cells with oil
C16.1	41.05 (0.45)	37.48 (2.23)
C16.0	18.32 (0.22)	23.60 (2.66)
C18.1	38.77 (0.48)	35.33 (2.95)
C18.0	1.88 (0.63)	3.39 (2.24)

Data are presented as mean percentage of total fatty acids from four replicate experiments; S.D. = standard deviation.

fluidity (Table 1). All these changes were statistically significant ($P<0.001$ to $P<0.003$). C18:1 fatty acids were considered to be unchanged with $P>0.5$ and <0.1. The fatty acid composition of microbial cell membranes affects their ability to survive in various environments (Ghfir et al., 1994). The ratio of saturated to unsaturated fatty acids can alter in response to environmental conditions (Odumeni et al., 1993). Maintenance of an optimal degree of fluidity of membrane lipids is important for normal function, with environmental adaptation known as homeoviscosic adaptation (Heipieper and de Bont, 1994, Rotert et al., 1993). An environment containing palmarosa oil increased the saturation of the yeast cell membrane, which would lead to changes in membrane potential and hence membrane channels.

In general, the primary site of the toxic action of terpenes is the plasma membrane, but the mechanism is poorly understood. Further, it is not clear yet if essential oils or their components also have an effect on the mitochondrial and vacuolar membranes. Studies using *Escherichia coli* liposomes have shown that the effect of terpenes on the structural and functional properties of biological membranes was directly related to accumulation in these membranes, the effect was independent of the structural features of the molecules (Sikkema et al.,

1994). Effects included swelling of the membrane bilayer, changes in membrane fluidity and increased passive flux of protons. Other studies have shown that terpenes have the ability to disrupt or penetrate lipid structures (Kararli et al., 1995; Takahashi et al., 1996; Williams and Barry, 1991). Monoterpenes, due to their lipophilic nature, diffuse into and damage cell membrane structures, partitioning from an aqueous phase into membrane structures (Andrews et al., 1980; Cox et al., 1998; Sikkema et al., 1995; Uribe et al., 1985).

Potassium is the most prevalent cation in the yeast cytoplasm (approx 150 mM) and is required for processes such as turgor maintenance, charge balancing and protein synthesis. There are several systems for its translocation. These include active transport via carrier permeases, counter transport with protons via H^+-ATPase and passive transport via potassium ion channels (Walker, 1998). Passive and facilitated diffusion across the plasma membrane has been described in detail (Rest et al., 1995). Highly selective voltage dependant potassium channels have been detected in the plasma membrane of *S. cerevisiae* (Gustin et al., 1986). The K^+ channels are active at both negative and positive membrane potentials (Ramirez et al., 1989). A small loss of K^+ can lead to the development of a large membrane potential (Navarro, 2000). Studies on K^+ efflux induced by cationic compounds in *S. cerevisiae* have shown that it is not evident that a voltage-dependant potassium channel is activated because no appreciable changes on membrane potential were observed (Enriquez-Freire et al., 1999). There are two separate transport systems in the plasma membrane for K^+ ions in S. *cerevisiae*: a high affinity system operated by an H^+/K^+ symport (Boxman et al., 1984), with a K_m value of 20 μM and a low affinity system with a K_m of 2 mM (Navarro and Ramos, 1984). An increase in the passive flux of protons or ions across the membrane impairs proper functioning of the membrane in energy transduction and screening the cytoplasm from the environment (Sikkema et al., 1995). In addition to the effect of the energy status of the cell, the maintenance of cellular homeostasis is affected by the increased membrane leakage.

Caesium blocks potassium channels in *S. cerevisiae* (Graves and Tinker, 2000). In the present study, potassium channels were blocked with 10 mM caesium ions prior to the oil treatment and potassium leakage was determined. No significant difference was observed between potassium leakage from *S. cerevisiae* treated with palmarosa oil and cells treated with caesium ions prior to the oil – K^+ ion leakage was 970 (±128) and 904 (±141) μmol/1.5×10^9 cells, respectively. This excludes the possibility of the ions leaking through potassium channels in the plasma membrane. The ionic radii of magnesium, potassium and calcium ions are 0.66, 1.33 and 0.99 Å, respectively. Our data suggest that the release of ions is not based on their size with only trace amounts of magnesium ions and no calcium ions leaking out, although they are smaller in size compared with the potassium ions confirming that the damage to the lipid bilayer of the plasma membrane is not due to formation of holes or lesions in it. Moreover, the fluidity of the cytoplasmic membrane is one important parameter in the maintenance of the homeostasis of the cell. The increased levels of saturated fatty acids and decreased levels of unsaturated fatty acids disorganized this fluidity. Further geraniol also appears to partition into membranes where it alters bilayer properties, making the membrane more fluid, increasing general permeability (Bard et al., 1988) leading to potassium loss from within the yeast cells. Our data showing no release of calcium ions from the treated yeast cells could be explained by the fact that most of the cellular calcium in *S. cerevisiae* is bound, precipitated or sequestered within the vacuole and Ca^{2+} homeostasis at low concentrations is maintained even under conditions when substantial Ca^{2+} efflux is induced (Eilam et al., 1984). Hence the vacuolar membrane seems to be unaffected.

While these effects are essentially overall membrane effects, the action of palmarosa oil on specific enzyme systems, especially membrane associated enzymes, should not be ruled out. One of the most abundant proteins in the yeast plasma membrane is the P-type H^+-ATPase that pumps protons out of the cells. The proton-pumping activity of the yeast cells is specifically blocked by inhibitors of the plasma-membrane ATPase, suggesting that the yeast plasma-membrane ATPase corresponds to the proton pump (Serrano, 1980). This essential enzyme plays a critical role in fungal cell physiology by maintaining the large transmembrane electrochemical proton gradient necessary for nutrient uptake and by regulating intracellular pH (Serrano, 1988). It is structurally and functionally related to the P-type Na^+, K^+-, H^+, K^+- and Ca^{2+}-ATPases of animal cells and the H^+-ATPases of plant cells (Ambesi et al., 2000).

In light of our data, it is proposed that the antimicrobial action of palmarosa oil takes place via a two-step process. The first step involves the passive entry of the oil into the plasma membrane in order to initiate membrane disruption. The second stage is the accumulation in the plasma membrane resulting in the inhibition of cell growth. This can be ascribed to the combined membrane effects such as increased bilayer disorder and ion leakage. These effects disturb the osmotic balance of the cell through loss of ions, make its membrane associated proteins inefficient due to increased membrane disorder eventually leading to inhibition of cell growth or cell death.

The volatility of palmarosa oil was demonstrated in control experiments in which palmarosa oil was added

to culture medium but without *S. cerevisiae*. A reduction of approximately 60% of the geraniol component was observed over 24 h as measured by GC. Evaporation of geraniol through the sponge plugs of the culture flasks was unavoidable if cells of *S. cerevisiae* were to remain aerobic. The actual concentration of the oil components affecting the yeast cells could not therefore be accurately determined for any of the experiments.

3. Experimental

3.1. General experimental procedures

Palmarosa oil was provided by Neal's Yard Remedies Ltd., Battersea, London.

3.2. Chemicals

Geraniol, geranyl acetate and caesium chloride, were obtained from Sigma and Triton X-100 from BDH. Other chemicals used were of analytical grade.

3.2.1. Media

Malt extract (Sigma), 3 g, yeast extract (Sigma), 3 g, glucose (BDH), 10 g, and mycological peptone (Oxoid), 5 g, were made up in 1 l distilled water and autoclaved at 121 psi for 15 min (MYGP broth). MYGP agar was made up as the broth with the addition of 20 g l^{-1} of Oxoid Agar No. 3.

3.3. Microorganism

The yeast used in this study was *Saccharomyces cerevisiae* CMI 61302 maintained at 4 °C on MYGP agar.

3.4. Identification of material (GC–FTIR analysis)

The GC analyses of the oils were performed using a Perkin-Elmer 8600 Gas Chromatograph with a 30 m × 0.75 mm SPB 35 column; injector and detector temperatures were 260 and 300 °C, respectively; oven temperature was 80–200 °C at 4 °C min^{-1} carrier gas was helium at a flow rate of 1.96 ml min^{-1}; amount injected was 0.2 μl (split ratio 10:1). Identification of the components in the oil was by GC–FTIR linked analysis using a Perkin-Elmer 1720 X FT-IR Spectrometer, with repeat scans. The IR spectrum of the component peak obtained from the GC trace was compared with that obtained from authentic samples.

3.5. Antimicrobial activity

Plates were prepared using 200 μl of culture broth of *S. cerevisiae* mixed with about 10 ml of molten MYGP agar. After allowing the plates to solidify, a 10 μl drop of palmarosa oil was placed in the centre of each plate and the plates incubated at 30 °C for 24 h, after which the diameter of inhibition of growth in mm was measured. With the components, a 6.5 μl drop of geraniol and a 2 μl drop of geranyl acetate were added. Volumes of the components used corresponded to their actual proportion in the oil. All experiments were repeated thrice, in triplicate, for each oil or component.

3.6. Metal ion leakage

Saccharomyces cerevisiae cells were grown in MYGP medium at 30 °C, 150 rpm to a cell density of 3×10^7 cells/ml ($OD_{600}=1$) in 250-ml Erlenmeyer flasks. Cells were then centrifuged at 6000*g* for 10–15 min, washed twice with 0.9% saline and resuspended in saline. The cells suspended in saline were then treated with the oil (0.1%) and their components at the concentration similar to that present in the oil. Every half hour for 2 h, cells were centrifuged and K^+, Mg^{2+} and Ca^{2+} ions were measured in the supernatant by atomic absorption spectroscopy using standard solutions of magnesium, potassium and calcium ions (0–15 ppm), as nitrates. Two control flasks were run in parallel each time, the first containing medium with cells but no oil and the second containing oil in saline (no cells). Data is presented in the form of experimental (treated cells) and control (untreated cells) readings. Minimally 24 replicates (number of replicates × number of readings per replicate) were performed for each oil or component. All error bars correspond to standard deviation.

3.7. Determination of total sequestered K^+

The cells were completely lysed in 3% Triton X-100, and the maximum sequestered K^+ that leaked from the cells was determined.

3.8. Blocking potassium channels

Saccharomyces cerevisiae cells were harvested, suspended in saline, then treated with 10 mM of caesium chloride and after an interval of 10 min, palmarosa oil (0.1%) was added, followed by incubation for 2 h. Estimation of the leaked potassium ions was quantified as before using atomic absorption spectroscopy. A control (cells + palmarosa oil) was run in parallel.

3.9. Analysis of membrane fatty acids

Cells were harvested by centrifugation at 4000 *g* for 5 min at 4 °C, washed in dist. H_2O thrice before freeze-drying for 24 h. Duplicates were prepared on two separate days to give a total of four replicates. Extraction of membrane fatty acids, preparation of their methyl esters and final extraction with ether was according to Rose

and Veazey (1988), but modified by sonication of cells under nitrogen at 14 MHz for 10 min (Soniprep 150, MSE) prior to alkaline saponification.

To 100 mg of sonicated freeze-dried cells was added 2 ml of 2 M KOH in 95% (v/v) methanol and 2 ml of benzene. The headspace was filled with N_2 gas and the mixture incubated at 80 °C for 3 h. On cooling an equal volume of methanol was added and the non-saponified fraction extracted by shaking with three successive 5 ml aliquots of petroleum ether (60–80° C fraction). The lower methanol layer was acidified with 6 M HCl and the saponified lipids were extracted with petroleum ether (three aliquots of 5 ml). The saponified lipid fraction was evaporated, then derivatised to methyl esters by addition of 1 ml of 14% BF_3 in methanol, the headspace filled with N_2 gas, sealed and heated at 80 °C for 1 h. The contents were added to 5 ml H_2O and extracted three times with 5 ml petroleum ether. The pooled ether fractions were concentrated and analysed by GC.

The GC was a Perkin-Elmer 8600 fitted with flame ionization detector. An SE30 phase (0.3 mm) column (45 m × 0.32 mm) was used with oven temperature at 70 °C.

Statistical analysis of data used the Kruskal–Wallis and/or *t*-test.

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