7.0 Microbiological and calorimetric investigations on the antimicrobial actions of different propolis extracts: an *in vitro* approach

7.1 Abstract

Propolis is a honeybee product which has been used by humans since ancient times as a multifaceted antimicrobial drug against several types of ailments. It had been, however, forgotten in the meantime due to the discovery and effective use of antibiotics. Nowadays the medicinal application of propolis is reviving mainly due to the emergence of pathogenic bacterial and fungal strains resistant to drugs, the undesirable side effects of some synthetic drugs, and also to the "back to nature" trend. Even though several publications exist in the realm of research on the antimicrobial actions of propolis, most of them are limited in scope by the use of only 1 type of extract, of samples from only 1 geographic location, and the inability to discern the mechanism of antimicrobial action.

In this chapter, the antibacterial action of 3 different types of extracts of propolis: (i) water-extracted propolis (WEP), (ii) propolis volatiles (PV), (iii) and ethanol-extracted propolis (EEP) were investigated by flow microcalorimetry coupled with polarography, and by Petridish bioassay methods. Antifungal investigations were performed only with the Petridish bioassay method because of difficulties with growing fungi in flow calorimeters. Antimicrobial activities were compared by the minimal inhibitory concentrations (MIC), minimal bactericidal concentrations (MBC), some calorimetric power-time curve characteristics, and the mean diameter of inhibition zone in Petridish bioassays.

The water-extracted propolis solution had the weakest antibacterial and antifungal action, compared to the other 2 extracts, which showed effects nearly similar to each other. The filamentous fungi were generally less sensitive to propolis than bacteria and yeasts, regardless of the types or concentrations of propolis. Treatment with some propolis samples at concentrations around or lower than MIC values, stimulated effluent growth of *Pseudomonas syringae*, a phenomenon called hormesis.

Flow microcalorimetric and polarographic investigations of the antimicrobial actions of propolis displayed its mechanism of action; both bacteriostatic and bactericidal actions were displayed depending on the concentration, type of propolis, and type of bacteria tested. The Gram negative *E. coli* was insensitive to most treatments, and higher concentrations of propolis, than for other bacteria, were required to achieve bactericidal effects.

Treatments of bacteria with weak propolis concentrations dropped the calorimetric power-time (*p*-*t*) curves to lower levels, where the curves remained for the rest of the

experimental period, or dropped to the baseline with the course of time, or revived after some time and attained peaks. The treatment with strong concentrations, however, dropped the curves to the baseline immediately.

7.2 Introduction

Propolis has been used by man since early times, for various purposes, especially as a medicine because of its antimicrobial properties (Crane 1990, Cheng and Wong 1996). Ancient Greek texts refer to the substance as a "cure for bruises and suppurating sore", and in Rome propolis was used by physicians in making poultices. The therapeutic properties of propolis (tzori, in Hebrew) are mentioned throughout the Old Testament. Records from 12th century Europe describe medical preparations using propolis for the treatment of mouth and throat infections, and dental cares (Krell, 1996). Several antimicrobial activities have been ascribed to propolis including antibacterial (Metzner et al. 1977, Grange and Darvey 1990, Ikeno et al. 1991, Kujumgiev et al. 1993, Aga et al. 1994, Digrak et al. 1995, Garedew and Lamprecht 1997, Menezes et al. 1997, Kujumgiev et al. 1999, Santos et al. 2002), antifungal (Pepeljnjak et al 1982, Dobrowolski et al. 1991, Digrak et al. 1995, Otta et al. 2001, Sawaya et al. 2002), antiprotozoan (Scheller et al. 1977, Starzyk et al. 1977, Dobrowolski et al. 1991, De Castro and Higashi 1995), and antiviral (König and Dustmann 1988, Amoros et al. 1992), among others.

Most of the hitherto antifungal investigations of propolis concentrated mainly on yeast, such as different species of *Candida,* or dimorphic human and animal pathogenic fungi (Lindelfelser 1967, Pepeljnjak et al 1982, Dobrowolski et al. 1991, Digrak et al. 1995, Otta et al. 2001, Sawaya et al. 2002) with satisfactory fungistatic and fungicidal results both *in vitro* and *in vivo* experiments. Nevertheless investigations on most saprophytic filamentous fungi are rare and fragmentary. The problem caused by fungi is not restricted to the realm of human and animal health. It is rather multifaceted, affecting different types of industry products such as paper and leather, among several others. The antimycotic property of propolis may thus be utilized in preventing the destruction of books and other valuable paper products in libraries and archives, and leather products by saprophytic fungi such as *Aspergillus sp., Penicillium sp.,* and *Trichoderma sp*., which are generally known for rotting and destruction of such products. For this reason one of the aims of the present investigation was to test the antimycotic property of propolis *in vitro*.

The medical use of propolis was nearly forgotten in modern era due to the discovery and effective use of antibiotics. Nowadays, however, since several pathogens are developing

resistance to potent antibiotics, and the latter causing side effects in humans, the need to search and screen for new antimicrobial agents is increasing (Walker 1996, Mc Devitt et al. 2002). This gave an impetus to several researchers to concentrate on research in potentially antimicrobial natural products such as propolis to add to our current arsenal. Because propolis is reputed to have several biological effects against various types of ailments, the list of pharmaceutical, health food and cosmetic preparations is increasing drastically (Burdock 1998).

Regardless of the increasing emergence of drug resistant microbes, the pace at which new antimicrobials are discovered and produced is slowing and the so called new and emerging pathogens are aggravating the problem (Russell 2002). The mechanisms of antimicrobial actions of antibiotics, and the resistance mechanisms by most microbes to antibiotics are well documented (Russell and Chopra 1996, Nikaido 1998). The mechanisms of action of biocides based on natural mixtures, such as propolis, are however, poorly understood due mainly to the several target sites they have within a bacterial cell (Denyer and Stewart 1998), and research to elucidate these mechanisms is inadequate. Therefore screening methods that contribute to the understanding of the mechanisms how propolis act are very important. However, as the need to isolate potent antimicrobial agents is increasing, several techniques that may not give a clue as to the mechanism of action of the biocide are employed in several laboratories. In addition to that, most standard antimicrobial screening methods are associated with drawbacks, and there is no standard way of presenting the results obtained with these methods (Rios et al. 1988). Few of the most common problems encountered with the majority of techniques employed today are: (i) different inoculum sizes used in various laboratories: this can affect results in both liquid and solid media directly, and different results could be obtained for the same concentration of an antimicrobial agent based on the density of microbes to be treated. This problem could be minimized to a certain degree by standardizing the inoculum density which is, however, laborious, and time consuming, (ii) consistency of the agar layer (activity of water): affects results by directly interfering with the diffusion potential of the compound being tested, (iii) incubation temperature: affects the diffusion potential of the substance being screened by acting on the consistency of the agar layer, (iv) polarity of the antimicrobial substance being tested: testing non-polar substances (like propolis) with the Petridish bioassay method is not as such credible since the substance could not diffuse properly through the polar agar layer, and hence antimicrobial activity could be highly restrained and restricted to a small diameter around the hole containing propolis solution, (v) colour of the substance to be tested: this interferes with results from the

spectrophotometric method. It could, however, be alleviated to a certain extent by using corresponding blanks for each dilution, but it can not be reliable. The problem with propolis is that, in addition to its natural colour which could interfere with absorbance by the bacterial cells, it forms a hazy suspension upon mixing with water due to the insolubility of most of its components in water that makes spectrophotometric measurement impractical.

A method which can not be affected by these problems is of interest in the search for antimicrobial agents. One of such methods that is robust enough to be used in the study of antimicrobial agents is calorimetry. This technique is applied in different fields of science with high precision and sensitivity (Lamprecht 1983). One of the aims of the present investigation is, therefore, to use calorimetry in the investigation of the mechanism of action of propolis and evaluate its credibility compared to the often-used standard microbiological methods in testing the effects of antimicrobials.

Research has been done on the biological activity of propolis against different sorts of ailments, infections and parasites in the past. But most investigations concentrated on a sample from 1 geographic location, 1 type of extract (usually the ethanol extract of propolis, EEP), or derivatives of 1 type of extract, and almost all used only the Petridish bioassay method with no, or very little hints about the mechanisms behind the antimicrobial effects. One of the purposes of the present investigations was to compare differences among the antimicrobial activities of propolis extracts from different geographic origins, and samples from the same apiary but different hives. The comparison between propolis samples requires considering the species and subspecies of bees that did the collection. The comparison between geographic samples will be done at the level of different extracts, i.e. ethanol-extracted propolis (EEP), water-extracted propolis (WEP), and propolis volatiles (PV).

7.3 Materials and Methods

7.3.1 Propolis acquisition and preparation of ethanol extracted propolis (EEP)

Propolis samples were obtained from different parts of the world by personal contact with beekeepers and scientists in the corresponding countries. The different propolis samples used in the investigations were tabulated in chapter 5 with their physical properties and the bees that collected them. According to the information obtained from the corresponding propolis suppliers, all propolis samples were collected by scrapping from frames and walls of the beehives. All samples were obtained as solid samples and extracted in 70% ethanol according to previously established and effective methods (see chapter 3). The extracts were cooled to 25 °C, and their electrical conductivity and pH were measured using a multichannel

WTW Multi 340i conductometer and pH meter (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). The propolis extracts were dried at 40 °C by monitoring the weight at an interval of 24 h starting from the 10^{th} incubation day. Complete dryness of the samples was ascertained by the absence of weight loss during the last 3 continuous weightings, and was achieved in about 15 days. The yield of extraction of each propolis sample was determined from the proportion of dry weight of extracted sample to that of fresh sample. The pH of the different propolis samples and yield of extraction are summarized in Table 7.1.

The dried propolis samples were dissolved in 60% ethanol to prepare 10% stock solutions, which were diluted further with the same solvent to achieve varying experimental concentrations. Though extraction of propolis was done with 70% ethanol, treatments were done with 60% ethanol to minimize the effect of a highly concentrated solvent.

7.3.2 Extraction of propolis volatiles (PV)

As it is often claimed that the volatile components of propolis are responsible for the lower density of aeroflora in the otherwise ideal bacterial and fungal flourishing beehive interior, the antimicrobial activities of the volatile components were investigated after extracting them. Due to limitation of sample size, antimicrobial activities of the volatile components of propolis (propolis volatiles, PV) were investigated only with samples from Colombia (C1), Ethiopia (E1), Germany (G1), Italy (I1), 2 samples from Poland (P1 and P2) and 3 samples from South Africa (SA1, SA3, and SA5).

The pre-weighed propolis samples were frozen at -20 °C to make handling of this highly sticky substance easier. The samples were then ground in an electric coffee mill and subjected to steam distillation for 4 h using a Lickens-Nickerson apparatus (Kujumjiev et al. 1999). The collected distillates were extracted with ethyl ether/n-pentane 1:1, and the extracts were dried over Na_2SO_4. The dried samples were weighed and dissolved in 60% ethanol to make a stock solution of 10% w/v (10 g/100 ml) for further use.

7.3.3 Water-extracted propolis (WEP)

Due to limitations of sample sizes obtained from different countries, it was not possible to prepare a water-extracted propolis solution of all samples. Such a solution was prepared only for the sample obtained from Berlin, Germany (G1). As the yield of extraction is very low compared to the ethanol-extracted propolis (see results), a large sample size was needed to get a reasonable amount of extract to perform experiments.

The extraction procedure used to obtain the ethanol-extracted propolis (section 7.2.1 above) was followed strictly except that the solvent here was distilled water instead of 70% ethanol. A 10% stock solution was prepared in distilled water and lower concentrations for treatments were prepared by diluting the stock solution with distilled water.

7.3.4 Biological material

Bioassays of the antimicrobial activities of the different propolis samples were performed using (i) 4 fungal species: the yeast *Saccharomyces cerevisiae* (DSM 211) and 3 filamentous fungi, *Aspergillus niger* (DSM 737)*, Penicillium chrysogenum* (DSM 844) and *Trichoderma viride* (DSM 63065); (ii) 4 species of Gram positive bacteria: *Bacillus subtilis* (DSM 347), *Micrococcus luteus* (DSM 348), *Bacillus megaterium* (DSM 90), *Bacillus brevis* (DSM 5609); and (iii) 2 species of Gram negative bacteria: *Escherichia coli* (DSM 31), *Pseudomonas syringae* (DSM 5176). All strains of microorganisms were bought from the German collection for microorganisms and cell culture (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - DSMZ, Braunschweig).

7.3.5 Growth media

Cultivation of all bacterial cultures was done in Standard I nutrient broth (Merck Lot VL 630582) and/or on Standard I nutrient agar (Merck, Lot VL 694681). The yeast was cultivated on a medium composed of 10 g Oxoid agar (Agar Bacteriological No.1, Lot 817706-2), 1 g yeast extract (Sigma, CAS No. 8013-01-2), 2 g glucose (Merck), 0.5 g peptone (Sigma, Lot 128H0184), 10 ml Na-phosphate buffer (1M, pH 7.0) in 1 l distilled water. The same medium without agar was used as nutrient broth for the yeast.

Trichoderma viride was cultivated on malt extract peptone agar (MEPA) composed of 30 g malt extract (Sigma, Lot 41k0181), 3 g soya peptone (Hy soy T, Sigma, Lot 128 H0184), 15 g Oxoid agar (Agar Bacteriological No.1, Lot 817706-2) in 1 l distilled water. *Aspergillus niger* and *Penicillium chrysogenum* were cultivated on potato dextrose agar (PDA) composed of 20 g glucose (Merck), 15 g Oxoid agar (Agar Bacteriological No.1, Lot 817706-2), in 1 l potato infusion obtained by boiling 200 g scrubbed and sliced potato in 1 l distilled water for 1 h and passed through a fine sieve, according to the recommendation of the DSMZ obtained up on purchase of the strains.

All types of media were sterilized by autoclaving at 121 °C and 1.03 bars for 15 min.

7.3.6. Petridish Bioassay

7.3.6.1 Agar well diffusion pour plate technique

The main purpose of this experiment was to compare the antimicrobial effects of the various propolis samples at levels of different concentrations. As the general procedures for inocula preparation and treatment are different, bacteria and yeasts are dealt in here separately from the filamentous fungi.

Propolis concentrations of 0.01, 0.10, 1.0, and 10.0% w/v were prepared by diluting the stock solution with 60% ethanol in case of EEP and with distilled water for WEP, and used in the antimicrobial tests. In the case of bioassays with propolis volatiles, experiments were done only with a 10% concentration for all samples due to sample size limitation.

Except for the WEP, where distilled water was used, the control for all experiments was 60% ethanol. Each experiment was repeated 3 times.

7.3.6.1.1 Bacteria and yeast

An isolated pure colony of an overnight grown culture was picked carefully using a sterile transfer loop, inoculated into a nutrient broth in an Erlenmeyer flask and grown overnight at 30 °C. A volume of 50 µl of the overnight culture was inoculated to a 20 ml nutrient broth and grown further for about 3 to 5 h until an O.D. of 0.6 (546 nm) was achieved. Optical density of the culture was measured using a spectrophotometer of the type UV 120-01 Shimadzu, Kyoto, Japan. The suspension was then diluted 1:50 with the corresponding nutrient broth in order to prepare the standard inoculum (method slightly modified after Faye and Wyatt, 1980).

The sterilized nutrient agar was cooled to 48 °C, 5 ml of the standard inoculum was mixed with 1 l nutrient agar and distributed into plastic Petridishes of ø = 85 mm, 10 ml in each, rendering an agar layer of 3.52 mm thickness. After the agar was solidified, 3 holes were bored per Petridish using a cork borer of ø = 9 mm. Each hole was then filled with 50 µl propolis solution and the Petridishes were placed in a refrigerator for 24 h, giving propolis sufficient time to diffuse. Finally, the plates were removed from the refrigerator, incubated at 30 °C for 24 to 48 h and the inhibition zones were measured. Antibacterial tests were done using propolis concentrations of 0.01, 0.1, 1.0, and 10.0 (% w/v).

7.3.6.1.2 Filamentous fungi

An isolated pure colony of a fungal culture, which was grown for 72 h on solid medium and started to sporulate, was scrubbed up using a sterile transfer loop and put into

sterile distilled water in a test tube. The hyphae were disintegrated by adding sterile glass beads and shaking vigorously for 1 min, in order to get a uniform suspension. The optical density of the suspension was measured at 546 nm and adjusted to 0.6. The suspension was then diluted 1:50 with sterile distilled water in order to prepare the standard inoculum. 5 ml suspension (standard inoculum) was added to a 1 l agar solution at 48 °C and the same procedure as above (section 7.3.6.1.1) was followed further until the final incubation phase. The plates were taken out of the refrigerator and incubated at 25 °C for 72 h and the inhibition zones were measured. Propolis concentrations of 0.1, 0.5, 1.0, 2.0, 4.0, 8.0, and 10.0 (% w/v) were tested, except for propolis volatiles with only 10%.

7.3.6.2 Agar dilution in plates

As the antimicrobial activity of propolis by the agar well diffusion pour plate method is highly influenced by the low hydro-solubility of biologically active components in the diffusion medium (the agar layer) estimation of the MIC values using this method may not be accurate (Sawaya et al. 2002). For this reason MIC values for the different propolis samples against the various bacterial and fungal species were determined by the agar dilution method, according to the recommendation of the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 1985).

Corresponding volumes of a 10% propolis solution or of lower concentrations were added to the sterile agar solutions at a temperature of 48 °C, to achieve final concentrations of 0.05, 0.1, 0.5, 1.0 1.5, and 2% w/v propolis in the fungal growth media and 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1% w/v propolis in the bacterial and yeast growth media. In addition to that final propolis concentrations of 1, 5 and 10% w/v were incorporated in the media for bacterial strains that were insensitive to the lower concentrations. The contents were mixed thoroughly, poured into sterile Petridishes, and allowed to cool. The same concentrations of propolis volatiles, as above, were tested against bacterial and fungal strains for their minimal inhibitory concentrations. Standard inocula were prepared for all bacteria, yeast and fungi following the same procedure as in the agar well diffusion pour plate method. Inoculation was done by pipetting 50 µl of the standard inoculum and uniformly distributing it on the surface of the propolis-containing agar layer using a sterile z-shaped glass rod. The plates were then incubated at a temperature of 30 °C for 24 h for bacteria and at 25 °C for 48 to 72 h for fungi. The minimal inhibitory concentration (MIC) was the lowest concentration of propolis that inhibited any visible growth of bacteria, yeast, or fungi.

7.3.7 Calorimetric Bioassay

All calorimetric experiments were performed with bacteria at a temperature of 30 °C using a flow calorimeter (Type 10700-1, LKB Bromma, Sweden) with a flow-through spiral of 0.587 ml. The calorimeter was connected by a Teflon tube of 1 mm inner diameter to an external fermenter, a 50 ml reaction vessel with 20 ml nutrient broth, placed in a water bath at 30 °C. The bacterial culture was circulated from the fermenter to the calorimeter and back using a peristaltic pump (Type LKB Pharmacia, Bromma, Sweden) at the outlet of the calorimeter in a sucking mode. The culture was vigorously stirred with a magnetic stirrer in order to avoid settling of cells and minimize depletion of oxygen in the fermenter and in the flow line.

7.3.7.1 Calibration of the flow calorimeter

As the sensitivity of the calorimeter and hence results could be affected, among several other factors, by the pumping rate, the calorimeter was electrically calibrated by pumping a phosphate buffer of pH 7 at pumping rates ranging from 1.5 to 100 ml h^{-1} and an experimental temperature of 30 °C. The sensitivity of the flow calorimeter decreases linearly with increasing pumping rate (see appendix Fig. A1).

To satisfy the contradicting requirements for a high flow rate (to avoid settling of cells and exhaustion of oxygen during the residence time in the tubing system) and a low one (to allow sufficient temperature equilibration of the culture before it arrives in the calorimetric spiral) a pumping rate of 56 ml h^{-1} was used for investigations with a prevailing aerobic metabolism. The sensitivity of the calorimeter at this pumping rate was 61.6 μV mW^{-1}.

7.3.7.2 Sterilization of the calorimetric set-up

The flow calorimetric line and the calorimetric spiral were sterilized by circulating a sterilizing solution composed of 10% H_2O_2 and 2% H_2SO_4 in 60% ethanol for 30 min before and after each experiment. After the allocated sterilization time the flow calorimetric set up was cleaned with 0.1 M potassium-phosphate buffer of pH 7.0 for 1 h. This sterilizing solution, in addition to sterilizing the calorimetric set up, washes out cells attached to the wall, and cleans any water insoluble propolis residue that could precipitate and settle on the inner wall of the tubes or the calorimetric spiral and subsequently block the flow system. Blockage of the flow-line by the settling of the water insoluble components of propolis was a very common phenomenon at low pumping rates and high cell densities after treatment with propolis. In order to avoid this problem a higher pumping rate, which reduces settling due to

the higher rate of streaming of fluid during the experiment, and the relatively aggressive cleaning and sterilizing solution that could dissolve and remove any precipitate from the tubing system were used.

7.3.7.3 Microcalorimetric cultivation of bacteria

As cultivation of moulds in a flow calorimetric setup is impossible due to the impracticality of pumping a broth containing a hyphal suspension, these calorimetric investigations were done only with bacteria. Even though it is in principle possible to cultivate yeasts in the flow calorimetric setup, like in the case of bacteria, it is very difficult and time consuming to deal with yeast cultures as they intend to cling to the inner wall of the tubing and block the flow system. For this reason flow calorimetric experiments with the yeast culture were avoided.

As recording of only the heat production rate may not explain what is happening in the tubing system and in the fermenter, oxygen consumption rate and the number of colony forming units (CFU) were recorded simultaneously for untreated, control bacterial cultures of all strains.

After the flow line was properly sterilized and cleaned with buffer, 20 ml of the growth medium (Standard I nutrient broth) were circulated for at least 30 min to establish a stable baseline. An inoculum of 200 µl of an overnight culture, grown on Standard I nutrient broth, of the experimental bacteria was then added and allowed to grow. Preliminary calorimetric experiments coupled with polarography and CFU count displayed that all strict aerobes, i.e. *B. megaterium, B. subtillis, B. brevis, M. luteus,* and *P. syringae*, have similar patterns of *p-t* curves, change in oxygen concentration in the flow line and of number of CFU, though minor differences exist among the shape of the *p-t* curves. The facultative anaerobe *E. coli*, however, showed a unique *p-t* curve due to the shift of metabolism to the anaerobic phase. For this reason, further calorimetric experiments were done using *E. coli* as a typical facultative anaerobe and *B. megaterium*, randomly chosen as a representative strict aerobe. The calorimetric signals were amplified (1000x) and recorded as power-time (*p-t*) curves by a two-channel recorder (BD5, Kipp and Zonen, The Netherlands). The recorded curves were digitalized using a Planimeter (Digikon DK 4261, Kontron Registriertechnik GmbH, München, Germany) and directly transferred to a PC. The ASCII data were then imported to statistic and graphic PC programmes for further processing. The results of the calorimetric experiments were presented as volume specific heat production rates (p in µW ml^{-1}) as a function of time.

7.3.7.4 Determination of O_2 concentration and CFU

The tube connection between the fermenter and the calorimeter, though it has to be kept as short as possible to avoid exhaustion of oxygen and hence the possible physiological change of the organism, is unavoidable. Though it is undesirable due to the mentioned facts, it however, provides space for the incorporation of electrodes in the flow line for the simultaneous monitoring of other culture characteristics, in addition to the heat production rate.

It is often mentioned that the concentration of oxygen decreases with increasing cell density, and becomes a limiting factor for bacterial growth and metabolism in the tubing system of the flow calorimeter. For this reason the concentration of oxygen was monitored by incorporating 2 galvanic oxygen electrodes, 1 in the fermenter, and the other in the flow line at the outlet of the calorimeter. The oxygen electrodes used were both of the type WTW Cellox 325, connected to WTW Multi 340i Data logger (Weilheim, Germany).

As the sterilizing chemicals were too aggressive to be used with the membrane oxygen electrodes, the latter had to be sterilized separately with only 70% ethanol. Due to the labour intensiveness of the procedure of dismantling the setup, sterilizing the electrode separately and assembling the flow-line with the oxygen electrode incorporated, this experiment was done only in few cases as a control. In addition to its labour intensiveness, the procedure of sterilizing the electrodes separately and assembling finally, increases the chance of contaminating the sterile flow line.

The number of CFU was determined by removing 50 µl culture from the outlet of the calorimeter (inlet of the fermenter) every 30 min. The samples were serially diluted and plated on Standard I nutrient agar, incubated for 24 h at 30 °C and the number of CFU was counted. Plates containing CFU < 30 were rejected in order to avoid possible experimental error.

7.3.7.5 Treatment of bacteria with propolis

Due to facts that will be dealt with in the discussion part of this chapter, it was decided to do the treatments only in the exponential phase of bacterial growth. In addition to that the effect of treatment at this phase is clearly visible by the drop in the level, or the change in the slope of the *p-t* curve, which is directly related to the growth/metabolic rate of the bacterial culture, and can be compared with other treatments. Treatment of bacteria with propolis was done in the exponential growth phase by adding 10, 25, 50 or 100 µl of the 10% ethanol-extracted or volatile components of propolis stock solution to the fermenter with 20 ml

bacterial culture, to achieve final propolis concentrations of 0.005, 0.0125, 0.025 or 0.05% (w/v). As the water-extracted propolis solutions were ineffective at these concentration levels, more volumes of the stock solution (10x) were added to the culture to achieve concentrations of 0.05, 0.125, 0.25 or 0.5% (w/v). The experiments with water-extracted and volatile components of propolis were done only with the sample G1 due to its sufficient availability.

In addition to the treatments at the exponential growth phase some experiments were also done by treating the culture with moderate concentrations of propolis after the calorimetric curve had dropped to the "calorimetric death phase".

The control experiments for each concentration of propolis were done by adding an equal volume of 60% ethanol, to observe the antimicrobial activity contributed by the solvent. Distilled water, instead of ethanol, was used as a double control in order to observe the influence on the heat production rate, if any, of the "dilution effect" of the liquid added rather than antimicrobial activity.

7.3.7.6 Determination of calorimetric MIC and MBC values

The minimum concentration of propolis that inhibited visible bacterial growth after incubation for a given period in a Petridish assay experiment was considered as the MIC value against the corresponding bacterial strains. In case of the calorimetric experiments, however, the minimum concentration of propolis that resulted in a drop of the *p-t* curve, was considered as the MIC value against the corresponding bacteria.

The minimum bactericidal concentrations (MBC) of the different propolis samples were also determined calorimetrically. The minimal concentration of propolis that killed bacteria and hence dropped the heat production rate to the baseline either immediately or first to a level above the base line and gradually, with incubation time, to the baseline were considered as the MBC.

7.3.8 Statistical analysis

Results were presented as mean ± S.D. values. Statistical tests were performed using the two-tailed student's t-test, paired sample t-test, 2-way ANOVA, 3-way ANOVA, and Tukey's post-hoc test, according to the nature of the data, and $\alpha = 0.05$ was taken as the critical value for all tests.

7.4 Results

7.4.1 Differences in the yields of extraction and physical properties of propolis samples

The pH of extracted propolis samples was acidic between 4.2 and 5.3, showing slight differences among different geographic samples, samples from the same geographic region and even from the same apiary. The pH was independent of the method of extraction as the 3 different types of extracts, i.e. EEP, WEP, and PV had similar pH values. The yields of the 3 extraction methods were highly variable; steam distillation extracted only the volatile components of propolis, and yielded 0.2% to 0.9% w/w, water extraction yielded 3.5% w/w, whereas the ethanol extraction method showed a very high yield of up to 61.3% w/w. There was also a high variation in the yield of extraction of the different propolis samples using the ethanol extraction method, with yields ranging from 10% for SA3 to 61.3% for P2. Variation was also observed in the yield of propolis volatiles (PV), which varied from 0.2% for SA1 and SA3 to 0.9% for G1. As the extraction of the water soluble components of propolis was done for only 1 sample, comparison between samples could not be done at this point. The yield of propolis showed dependency on the quality of raw propolis. Those propolis samples that were pure and sticky when they were still raw had higher yields. However, even though C1 and P4 had impurities, they demonstrated higher yields; whereas even though K1 was pure propolis it had a lower yield compared to the other pure propolis samples (Table 7.1).

Table 7.1 Physical properties and yield of extraction of the propolis samples
Yield (% w/w) of EEP (ethanol-extracted propolis), of WEP (Water-extracted propolis), and of PV (propolis volatiles). Electrical conductivity (C) in μS cm^{-1}, pH value, and description of the raw propolis quality. (The conductivity values are after correction for the corresponding solvents since distilled water and 60% ethanol have different conductivities, as ethanol is a non electrolyte.)

Sample name	EEP			PV			Remarks
	pH	C	yield	pH	C	yield	
C1	4.0	22.3	35.7	4.1	15.3	0.6	Impure propolis, slightly sticky
E1	5.3	25.4	44.7	5.0	12.4	0.8	Pure propolis, very sticky
G1	4.4	21.2	56.2	4.4	16.3	0.9	Pure propolis, very sticky
K1	4.8	33.4	31.2	-			Pure propolis, very sticky
Rus1	4.9	16.7	55.0	-			Pure propolis, very sticky
P1	4.8	44.2	54.5	4.9	22.3	0.5	Pure propolis, very sticky
P2	4.7	26.6	61.3	4.6	11.6	0.6	Pure propolis, slightly sticky
P3	4.7	24.2	59.3				Pure propolis, very sticky
P4	4.9	15.4	47.9				Impure propolis, slightly sticky
SA1	5.2	51.2	16.4	5.0	25.8	0.2	Impure propolis, slightly sticky
SA3	5.1	22.6	10.6	5.3	10.3	0.2	Impure propolis, slightly sticky
SA5	4.9	29.3	22.6	4.6	11.2	0.3	Impure propolis, slightly sticky
SA6	4.5	17.4	24.8				Impure propolis, slightly sticky
SA8	5.0	42.3	22.7				Impure propolis, slightly sticky
SA11	4.6	33.6	13.1				Impure propolis, slightly sticky
I1	4.9	36.5	55.3				Pure propolis, very sticky
WEP	4.3	98.9	3.5				Pure propolis, very sticky

7.4.2 Antimicrobial activities of ethanol extract of different propolis samples

Comparisons of the antimicrobial activities of the various propolis samples against the different test organisms were done at several levels of propolis concentrations using the diameter of inhibition zone as a parameter. A 3-way analysis of variance ANOVA ($\alpha = 0.05$) followed by the Tukey's post-hoc test using the variables: bacterial strain, propolis type, and concentration of propolis displayed that significant differences exist ($p < 0.05$) between the antibacterial activities of different concentrations within a sample, the activity increasing with increasing concentrations above the MIC values for each. However, this trend was not always observed. No statistically significant difference existed ($p > 0.05$) in some cases in the antifungal activities of the different concentration of a propolis sample, i.e. the diameter of the inhibition zone remained almost constant as the concentration changed stepwise from 1% to 10% (see appendix, Table A2).

Considering the antibacterial activity of the various propolis samples against a bacterium at a certain concentration level, it becomes clear that the samples showed antibacterial activities slightly different from each other, but no statistically significant difference, regardless of their geographic origins (see appendix, Table A1).

The slight differences observed in the antimicrobial activities of the different propolis samples could also not be explained based on the species of bees that collected them, since the difference within a species could be as high as or even higher than that between species. The samples SA1 to SA11 were collected by *Apis mellifera capensis* (South Africa), E1 by *A. mellifera scutellata* (Ethiopia), P1 to P4 by *A. m. mellifera* (Poland), G1 by *A. m. carnica* (Germany), I1 by *A. mellifera ligustica* (Italy), C1 by *Tetragonisca angustula* Illger (stingless bee) (Colombia).

7.4.3 Comparison of antimicrobial activities of different extracts

Comparison of the 3 different groups of extracts, i.e. ethanol-extracted propolis (EEP), water-extracted propolis (WEP) and the propolis volatiles (PV), was done by considering the minimal inhibitory concentrations (MIC) (Tables 7.2, 7.3, 7.4) and the inhibition diameter of a 10% propolis sample (see appendix Tables A1, A2 and A3). All propolis samples were extracted with 70% ethanol and thus had an ethanol-extracted propolis component. But due to sample size limitation, PV extracts were prepared only for C1, G1, E1, I1, P1, P2, SA1, SA3 and SA11 and WEP was obtained only from G1.

Table 7.2 Minimal inhibitory concentrations (MIC) of propolis against bacteria
MIC values (% w/v) of EEP from different geographic origins, and of a WEP, against bacterial species, determined by the agar dilution on plate method. n.d. indicates that no inhibitory concentration was detected in the range tested, up to 10% w/v.

Propolis type	*B. brevis*	*B. megaterium*	*B. subtilis*	*M. luteus*	*E. coli*	*P. syringae*
WEP	0.500	10.000	10.000	10.000	n.d.	10.000
I1	0.010	0.010	0.060	0.100	0.500	0.060
E1	0.005	0.060	0.040	5.000	n.d.	0.080
C1	0.040	0.040	0.040	0.500	n.d.	0.080
K1	0.005	0.010	0.080	0.060	0.100	0.060
G1	0.010	0.010	0.040	0.040	0.100	0.500
RUS1	0.010	0.010	0.020	0.020	5.000	0.040
P1	0.010	0.010	0.040	0.080	0.100	0.040
P2	0.010	0.010	0.040	0.500	0.100	0.500
P3	0.040	0.010	0.040	0.060	0.100	0.100
P4	0.060	0.010	0.040	0.060	0.100	0.100
SA1	0.005	0.010	0.005	0.020	0.100	0.005
SA3	0.005	0.060	0.005	0.040	5.000	0.060
SA5	0.005	0.005	0.005	0.005	1.000	0.005
SA6	0.005	0.005	0.005	0.005	5.000	n.d.
SA8	0.005	0.005	0.005	0.060	1.000	0.010
SA11	0.005	0.060	0.060	0.080	1.000	0.040

Table 7.3 Minimal inhibitory concentrations (MIC) of propolis against fungi
MIC values (% w/v) of EEP from different geographic origins, and of a WEP against various filamentous fungi and a yeast determined by the agar dilution on plate method. n.d. indicates that no inhibitory concentration was detected in the range tested, up to 10% w/v.

Propolis type	*T. viridae*	*A. niger*	*P. chrysogenum*	*S. cerevisae*
WEP	n.d.	n.d.	n.d.	5.00
I1	1.00	1.00	1.00	0.10
E1	n.d.	2.50	1.00	0.50
C1	n.d.	0.50	1.50	0.10
K1	0.50	0.50	0.50	0.04
G1	1.50	1.50	1.50	0.10
RUS1	2.00	1.00	1.50	0.50
P1	1.00	1.50	1.00	0.04
P2	1.00	1.00	0.50	0.50
P3	0.50	1.00	0.50	0.04
P4	0.50	1.00	0.50	0.10
SA1	n.d.	1.00	1.00	0.10
SA3	n.d.	1.00	1.00	0.50
SA5	10.00	1.00	1.00	0.06
SA6	n.d.	1.00	1.00	0.08
SA8	2.00	1.00	1.00	0.01
SA11	2.00	1.00	1.50	0.50

The water-extracted propolis (WEP) was shown to be significantly less active ($p < 0.05$, t-test) than the ethanol-extracted one from the same apiary (G1) as displayed by the higher MIC value and also by the smaller diameter of inhibition zone achieved by the 10%

WEP against each organism tested. Inferiority of the antimicrobial action of WEP of G1 also holds true when compared to the ethanol-extracted propolis samples obtained from different geographic regions.

Table 7.4 Minimal inhibitory concentrations (MIC) of propolis volatiles (PV)
MIC values (% w/v) of propolis from different geographic origins against various bacterial and fungal species determined by the agar dilution on plate method. n.d. indicates that no inhibitory concentration was detected in the range tested, up to 10% w/v.

	E1	C1	G1	P1	P2	SA1	SA3	SA5	I1
B. brevis	0.01	0.10	0.08	0.04	0.04	0.01	0.01	0.01	0.08
B. megaterium	0.10	0.10	0.08	0.04	0.04	0.04	0.10	0.04	0.08
B. subtilis	0.08	0.10	0.08	0.08	0.08	0.01	0.01	0.01	0.08
M. luteus	n.d.	1.00	0.08	0.10	1.00	0.06	0.08	0.02	0.08
E. coli	n.d.	n.d.	0.50	0.50	0.50	0.50	n.d.	5.00	0.50
P. syringae	0.10	0.10	1.00	0.06	1.00	0.01	0.01	0.01	0.50
S. cerevisae	1.00	0.50	0.50	0.08	1.00	0.50	1.00	0.10	0.50
A. niger	5.00	1.00	5.00	2.50	2.50	5.00	5.00	5.00	2.50
P. chrysogenum	5.00	2.50	5.00	2.50	2.50	5.00	5.00	5.00	2.50
T. viridae	n.d.	n.d	5.00	2.50	2.50	n.d.	n.d	n.d	n.d.

A 10% concentration of the volatile components of propolis (PV) showed slightly, but not statistically significant ($p > 0.05$, t-test) weaker antimicrobial activities than the ethanol-extracted one, against each microbe (cf. the 10% inhibition columns of Table A1 and A2 with that of Table A3 in the appendix). The t-test for statistical significance was performed for each pair of extracts i.e., EEP versus PV of each propolis sample against every bacterial and fungal species tested. All PVs showed significantly higher antimicrobial activities compared to the WEP of the sample G1.

The minimal concentrations of propolis needed to inhibit microbial growth were higher in case of the PVs than the EEPs. A 2 to 10 fold concentrated PV was needed in order to get a complete inhibition of bacterial growth as would be achieved by the EEP of the same propolis sample (cf. Tables 7.2 and 7.3 with Table 7.4). The filamentous fungi were less sensitive or even insensitive to the volatiles components of propolis at lower concentrations, as in the case of the ethanol extracts of propolis. The sensitivity of bacteria and fungi demonstrated by the diameter of the inhibition zone was not clearly segregated at a higher PV concentration (10% w/v), except for *B. brevis* which showed significantly higher inhibition zone for all PV samples tested. Bacteria that were sensitive to only highly concentrated ethanol extracts of propolis (*M. luteus* to 5% E1 and *E. coli* to 5% SA3) were insensitive even to a 10% PV.

7.4.4 Differences in the sensitivity of various microorganisms to propolis

Comparison of sensitivity of the different test organisms in view of the MIC values displayed in Tables 7.3 and 7.4, and also of the inhibition diameters displayed by higher concentrations (see appendix Table A1 and A2), demonstrate that filamentous fungi are generally less sensitive to propolis treatment. The minimal inhibitory concentrations of the various propolis samples against the filamentous fungi tested lies between 0.5 and 2.5% w/v propolis (Table 7.3), and 0.005 to 0.5% w/v against the different bacteria except *E. coli* (Table 7.2).

The yeast showed significantly higher MIC values than most bacteria, but significantly lower ones than the 3 moulds. Nevertheless, at 10% w/v the inhibition zones were similar to that obtained for most bacteria. The 4 Polish (P1 to P4) and the Kazakh (K1) propolis samples showed very large inhibition zones against the yeast *S. cerevisae*, significantly higher than that achieved for any other organism tested.

Among the bacteria, the Gram negative bacterium *E. coli* was highly resistant to propolis treatments followed by the other Gram negative *P. syringae* (see appendix, Table A1). *E. coli* did not show any recognizable response to the 10% treatments with E1, C1 and WEP, and small inhibition diameters with the other propolis samples. Only the Russian and Kazakh propolis produced larger inhibition diameters (see appendix, Table A1).

In the agar well diffusion pour plate method, treatments with propolis concentrations lower than the MIC values stimulated dense growth of *P. syringae* around the hole containing propolis (Fig. 7.1). Concentrations slightly higher than the MIC values produced a small inhibition zone around the hole, usually surrounded by a zone of dense bacterial growth (Fig 7.3 a and b, and see appendix Table A1). No encouragement of bacterial growth was observed at higher concentrations of propolis where only inhibition zones of different diameters were observed. This phenomenon of encouragement of bacterial growth at concentrations around the MIC values was not observed for any other organism tested, it was a unique feature observed for *P. syringae*.

The 2 moulds *A. niger* and *P. chrysogenum* showed similar sensitivities to the treatments with all propolis samples. However, *T. viridae* was insensitive to 3 of the 6 South African samples (SA1, SA3 and SA5), to the sample from Ethiopia (E1), from Colombia (C1) and the water-extracted propolis (WEP) at a 10% concentration. All filamentous fungi were insensitive to 10% WEP

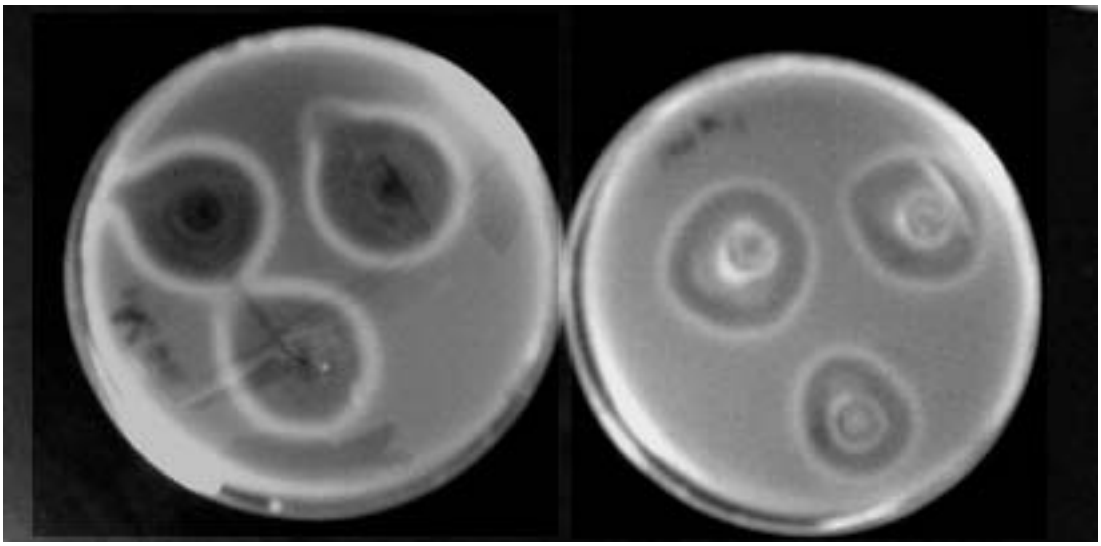


Fig. 7.1 Hormesis effect of a 0.1% E1 (left) and a 0.01% SA1 (right) EEP on *P. syringae.* The grey spots at the centre of the inhibition zones (in the well) are propolis precipitates, which did not diffuse due to hydrophobicity.

7.4.5 Calorimetric experiments

7.4.5.1 Calorimetric cultivation of bacteria

Since the patterns of curves of the heat production rates, oxygen concentration in the flow line and in the fermenter, and the number of colony forming units (CFU) were similar for all strict aerobes, treatments were done only with *B. megaterium* as a representative of them and with the facultative anaerobic *E. coli,* with a unique *p-t* curve. For this reason further calorimetric discussions and illustrations will mainly concentrate on these 2 bacterial strains. Even though the *p-t* curves of the strict aerobes investigated have similar patterns, they are not actually exactly alike since differences exist in the level at which the peak was achieved, 0.45 μW ml^{-1} for *B. megaterium*, 0.73 μW ml^{-1} for *B. brevis*, 0.52 μW ml^{-1} for *M. luteus*, 0.58 μW ml^{-1} for *B. subtillis*, and 0.57 μW ml^{-1} for *P. syringae.* In addition to that, differences also exist in the slope of ascend of the *p-t* curves, and slope of descend of the curve after the peak, and level of heat production rate at the calorimetric death phase. But the curves from the different strict aerobes have basic resemblances in shape, with room for minor differences in the features mentioned above due to specific features of each strain.

The microbial metabolic and growth events taking place in the fermenter during the first few hours of growth were represented by an initial lag phase followed by an exponential rise of the heat production rate and of the number of CFU. These events continued similarly up to the peak of heat production rate. After the *p-t* peak (0.45 μW ml^{-1}) for *B. megaterium*, the heat production rate dropped steeply whereas the number of CFU increased until to the

stationary phase with a cell density of 1.47 x 10 ^{8} CFU ml^{-1}, about twice as much as that at the peak (7x 10 ^{7} CFU ml^{-1}) about 2 h before. The heat production rate then remained at a lower level and the number of CFU at a higher level (Fig. 7.2 i). In case of *E. coli,* however, the nature of the *p-t* curve showed a different pattern. After the *p-t* curve achieved its peak at 0.62 µW ml^{-1}, it dropped down like in the case of the other bacteria but only to a level of about 0.21 µW ml^{-1}. After this point the rate of heat production rate increased and the curve started to ascend again until it achieved a level at about 0.5 µW ml^{-1}, lower than the first aerobic peak at 0.62 µW ml^{-1}(Fig. 7.2 ii) and remained at this level for the rest of the experimental period. It then started to drop gradually when the experimental period was extended for 10 to 15 h.

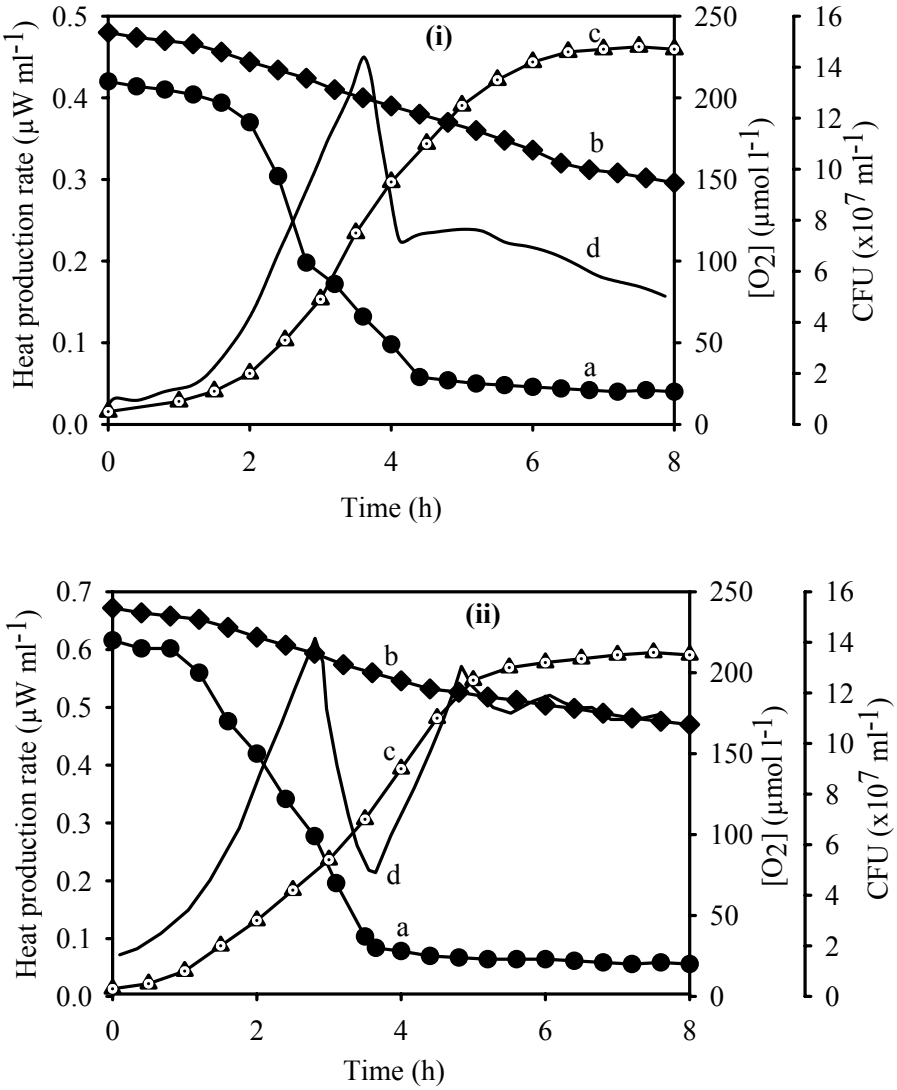


Fig. **7.2** Simultaneous recording of (a) oxygen concentration in the flow line, (b) oxygen concentration in the fermenter, (c) the number of colony forming units (CFU), and (d) the heat production rate of untreated cultures of **(i)** *B. megaterium* and **(ii)** *E. coli* in a flow microcalorimetric experiment.

The simultaneous monitoring of oxygen concentration in the flow line and in the fermenter displayed a big disparity between the two at higher cell densities in the middle and late experimental periods. At lower cell densities, at the lag phase and early exponential phase of bacterial growth, the concentration of oxygen in the flow line and in the fermenter were roughly similar, the latter showing a slightly higher concentration, by about 30 μmol l^{-1}. The beginning of the exponential growth was marked by an increase in the difference of the concentration of oxygen between that in the fermenter and in the flow line. The concentration of oxygen in the fermenter decreased gradually from 230 μmol l^{-1} at the lag phase to 152 μmol l^{-1} at the stationary phase. The concentration of oxygen in the flow line, however, dropped steeply from 200 μmol l^{-1} at the beginning of the exponential growth phase to 25 μmol l^{-1} shortly after the heat production peak was achieved. The peak of the calorimetric curve was achieved at an online oxygen concentration of 50 to 60 μmol l^{-1} and then declined with the steeply declining oxygen concentration for both *B. megaterium* and *E. coli*. The pattern of change of the oxygen concentration in the flow line and in the fermenter for *E. coli* and the other bacteria were alike regardless of the nature of the *p-t* curves (Fig 7.2 i and ii).

7.4.5.2 Effect of propolis treatment on bacterial culture properties

In order to evaluate the change in bacterial culture properties after treatment with lethal and sublethal doses of propolis, *B. megaterium* was treated with 0.025% and 0.05% w/v SA8, and *E. coli* with the same concentrations of P3.

After treatment of *B. megaterium* with 0.025% SA8, the heat production rate dropped suddenly from 0.39 to 0.15 μW ml^{-1} (61.5%) and the concentration of oxygen in the flow line and fermenter rose suddenly from 102 to 182 μmol l^{-1} and from 205 to 230 μmol l^{-1}, respectively. The number of CFU, however, dropped relatively slowly from 9.1 x 10^7 CFU ml^{-1} to 5.5 x 10^7 CFU ml^{-1} (Fig. 7.3 i). Following the drop in the heat production rate and CFU to lower values both stayed at plateau levels for nearly 2 h and then started increasing. The heat production rate increased further and achieved a peak at 0.42 μW ml^{-1} slightly lower than the peak of a control experiment (0.45μW ml^{-1}). The oxygen concentrations in the fermenter and in the flow line dropped with different rates, the one in the flow line dropping faster to a value of 25 μmol l^{-1} as the *p-t* curve dropped to a minimal value due to anaerobiosis. The online oxygen concentration, at which the *p-t* curve achieved its peak after treatment with the sublethal propolis dose, was 68 μmol l^{-1}, slightly but not significantly higher than the control experiments, 50 to 60 μmol l^{-1}.

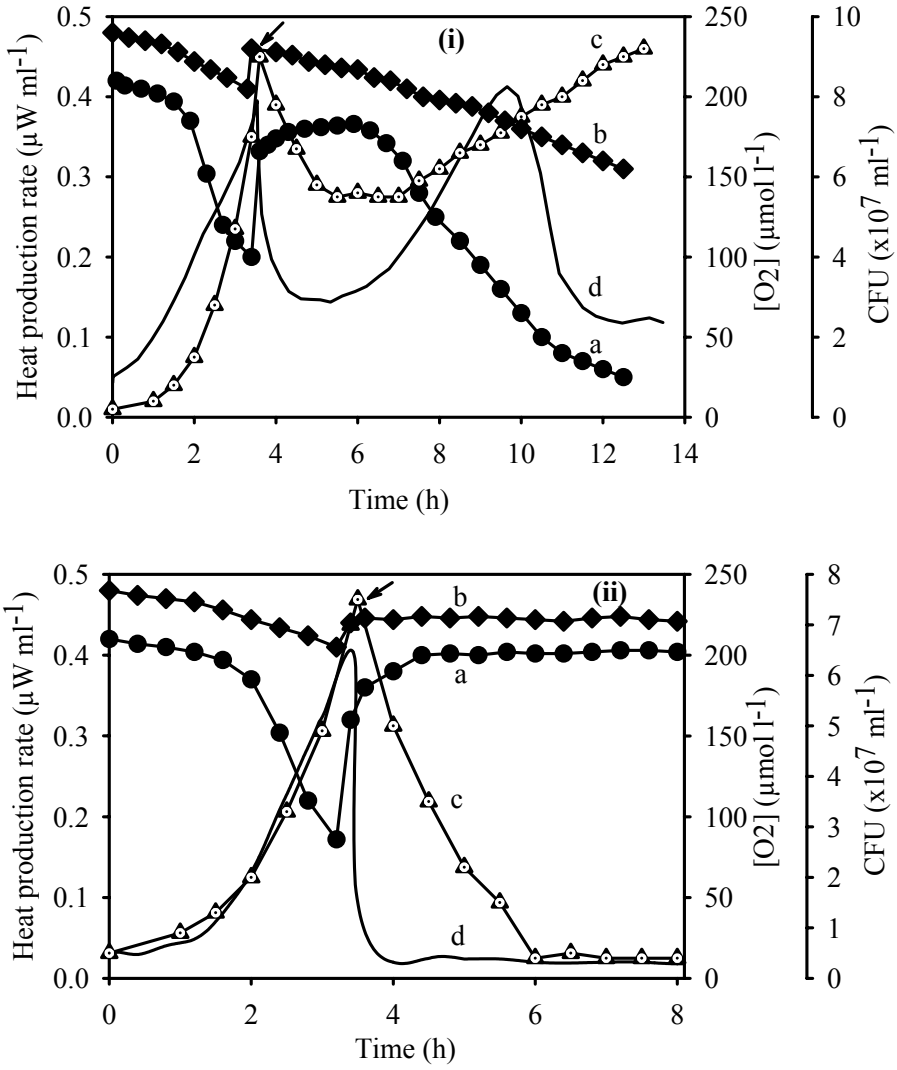


Fig. 7.3 Simultaneous recording of (a) oxygen concentration in the flow line, (b) oxygen concentration in the fermenter, (c) the number of colony forming units (CFU), and (d) heat production rate of a culture of *B. megaterium* treated with **(i)** 0.025% and **(ii)** 0.05% EEP of SA8 in a flow microcalorimetric experiment. Arrows indicate treatment.

After the *p-t* curve of *B. megaterium* dropped drastically due to the treatment of the culture with a lethal dose, it remained at about 0.03 µW ml^{-1} with a corresponding CFU of 4.5 x 10^6. Whereas the concentration of oxygen rose and remained at a higher level, at 198 µmol l^{-1} and 223 µmol l^{-1} in the flow line and in the fermenter, respectively (Fig. 7.3 ii). Unlike the changes in the rates of heat production and oxygen concentration in the flow line and in the fermenter, which are sudden, the number of colony forming units dropped gradually with incubation time, until it achieved its minimal level. *E. coli* responded similarly to the treatments with sublethal and lethal doses of propolis, as shown in Fig. 7.4 i and ii.

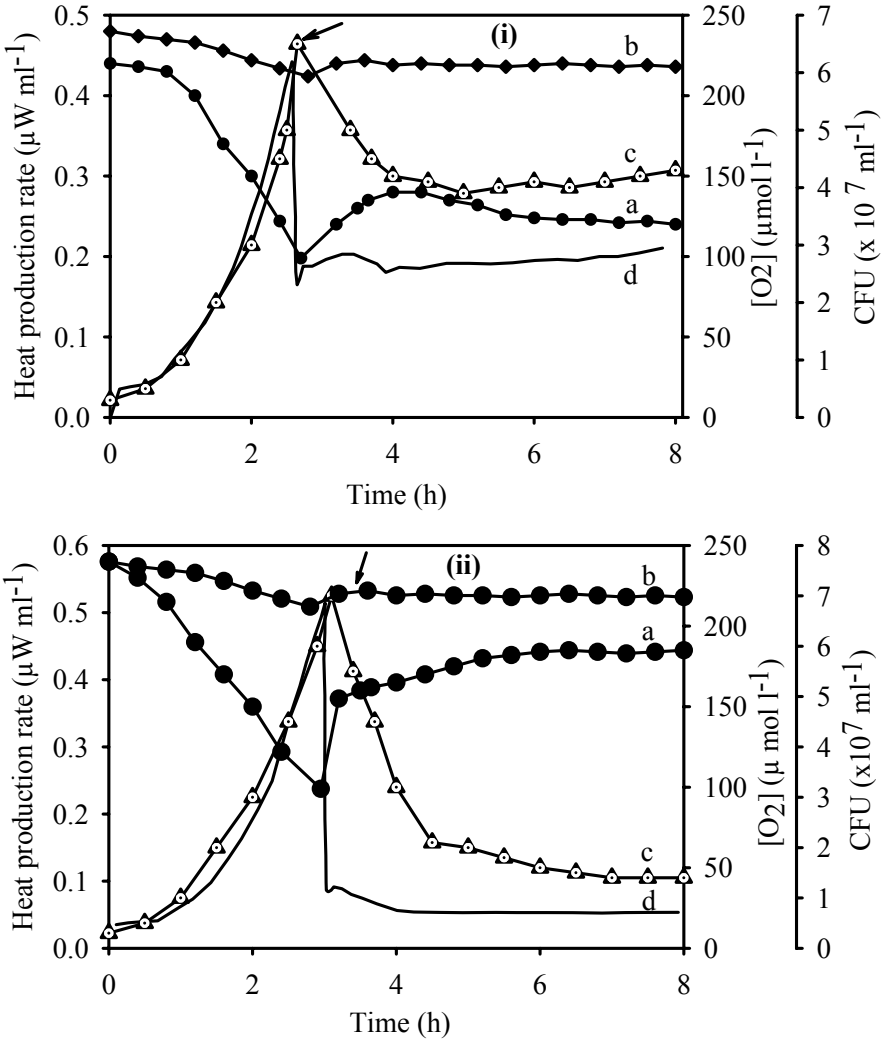


Fig. 7.4 Simultaneous recording of (a) oxygen concentration in the flow line, (b) oxygen concentration in the fermenter, (c) the number of colony forming units (CFU), and (d) heat production rate of a culture of *E. coli* treated with **(i)** 0.025% and **(ii)** 0.05% EEP of P3 in a flow microcalorimetric experiment. Arrows indicate treatment.

Treatment of *B. megaterium* with 0.025% EEP of SA8 after the *p-t* curve and the online oxygen concentration dropped to the minimal values, displayed that the 2 curves rose and attained values of 0.35 μW ml^{-1} and 147 μmol l^{-1}, respectively (Fig. 7.5). These increases were accompanied by corresponding increases in oxygen concentration in the fermenter and a slight decrease in the number of CFU. The curves then returned to the same level as before treatment in a short period of time.

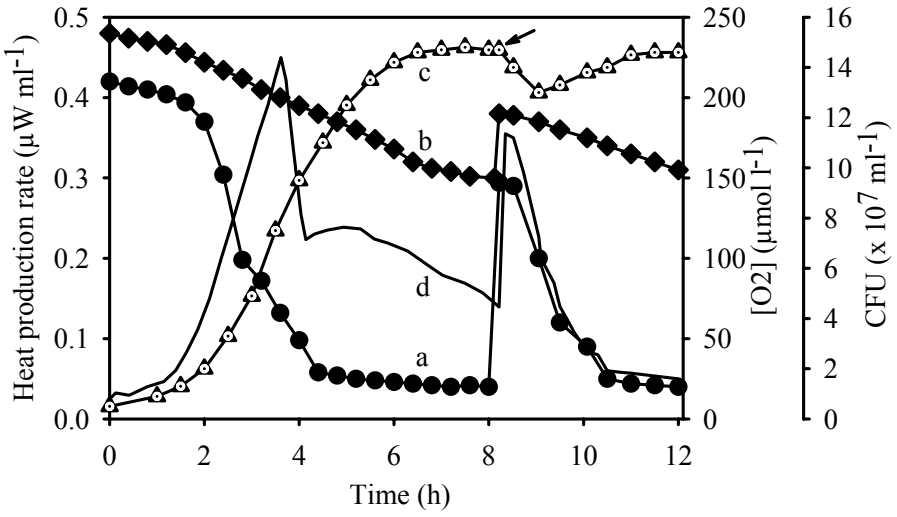


Fig. 7.5 Simultaneous recording of (a) oxygen concentration in the flow line, (b) oxygen concentration in the fermenter, (c) the number of colony forming units, and (d) the heat production rate of a culture of *B. megaterium* treated with 0.025% EEP of SA8 at the calorimetric death phase in a flow microcalorimetric experiment. The arrow indicates treatment.

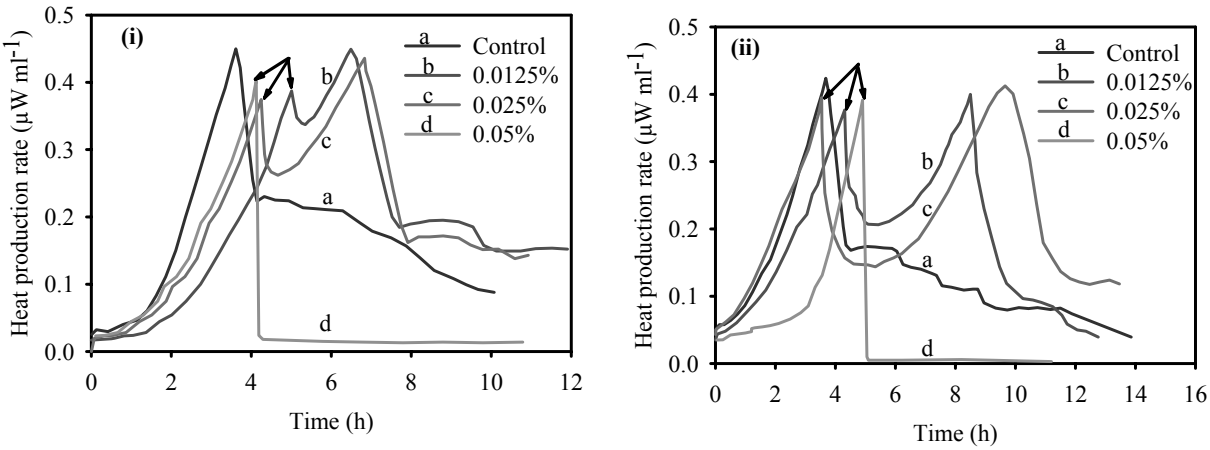
7.4.5.3 Calorimetric dose-response curves

Comparison of the antibacterial activities of the different propolis samples were done calorimetrically by treating an exponentially growing culture of *B. megaterium* in a flow calorimeter with different concentrations of propolis from various geographic origins. The calorimetric curves after treatment of the culture with propolis (Fig. 7.6 i and ii) provided several information as to the kinetics of action of the different propolis samples. The following culture and curve after treatment-parameters were used in comparison with those before treatment, and/or of a control culture: (i) MIC and MBC, (ii) level of the *p-t* curve after treatment, (iii) slope of curve ascend after treatment, and (iv) peak of the *p-t* curve achieved after treatment.

7.4.5.3.1 Calorimetric MIC and MBC values

The minimum concentration of propolis that affected the *p-t* curve by dropping it to a level lower than that before treatment, regardless of the extent of drop, was considered as the minimal inhibitory concentration (MIC) of propolis for that specific sample. The minimal concentration of propolis that dropped the curve to the baseline due to killing of bacteria and retained it at that level for the rest of the experimental period (8 to 10 h) was considered as the minimal bactericidal concentration (MBC). In addition to the immediate drop of the curve to the baseline due to treatment of the culture with MBC, few cases were observed where the

curve dropped first by 80 to 90% of the initial level and started to decline gradually and reached the baseline in a short while, after 1 to 2 h. Such propolis concentrations were also



considered as the minimal bactericidal concentrations for the corresponding samples.

Fig. 7.6 Effect of different concentrations of EEP from (i) Poland (P3) and (ii) South Africa (SA8) on the structure of the *p-t* curve of the bacterium *Bacillus megaterium*. Arrows indicate the time of treatment.

In cases where the treatment of a culture with a certain concentration of propolis accelerated the microbial growth and increased the slope of ascend of the curve after treatment significantly (Paired sample t-test, $\alpha = 0.05$), compared to that before treatment, it was not categorized as MIC. Such a concentration was considered as that which encourages microbial growth through a hormesis effect. If a treatment with a weak concentration of propolis did not result in a drop in the level of the *p-t* curve and if the slope of ascend of the curve after treatment was not significantly different from that before treatment, the corresponding concentration was considered to have no effect. The MIC and MBC values for the different propolis samples were determined according to these criteria and are summarized in Fig. 7.7.

The MIC values of most ethanol-extracted propolis samples lay at about 0.005% w/v with the exception of 5 of the 6 South African samples and the samples from Russia (Rus1) and Germany (G1) that showed a MIC value of 0.0125% w/v. The MBC value for most propolis samples was 0.05 % w/v with few exceptions. The sample from Kazakhstan killed bacteria and dropped the curve to the baseline at a concentration 4x weaker than most other samples, even though it had the same MIC value as others. The weakest propolis samples regarding the MBC value were the sample from Russia and 2 from South Africa. These 3 samples already showed relatively higher MIC values.

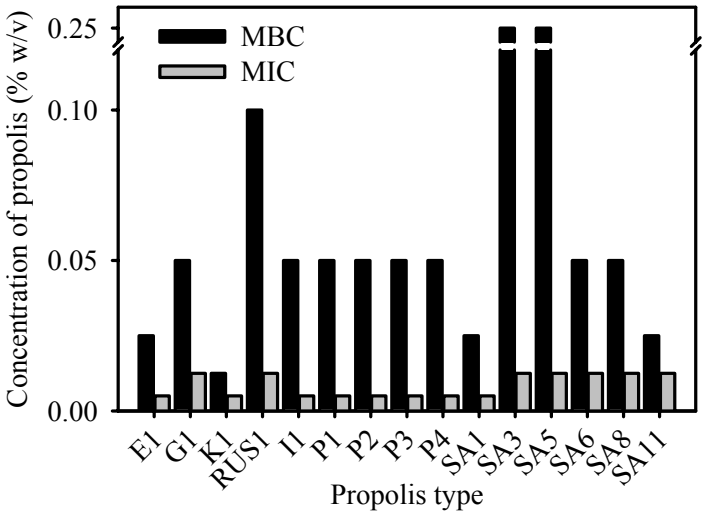


Fig. 7.7 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of different propolis samples against *B. megaterium*, determined calorimetrically.

7.4.5.3.2 Level of the *p-t* curve after treatment with different concentrations of propolis

The treatment of an exponentially growing bacterial culture with a concentration of propolis ≥ MIC resulted in a drop of the *p-t* curve to a lower level. Based on the type and concentration of propolis, the curve then either stayed at that level for a certain period of time, and then ascended to achieve a second peak or gradually dropped to the baseline, due to bacterial death (Fig. 7. 6 i and ii). By measuring the vertical distance between the point on the *p-t* curve at which treatment was done and the lowest point achieved on the curve due to the treatment of the culture, the dose-response relations for the different concentrations of propolis from various geographical origins were summarized in Fig. 7.8. The dose response curve for each propolis sample is typical, increasing the level of drop in the heat production rate due to treatment with growing concentration of propolis. As observed for all propolis samples, if the level of the curve was reduced by 80 to 100 %, it did not recover, and no second peak was observed. However, except for the sample from Kazakhstan (K1) that initially dropped the curve to 65% of the original value which gradually descended to the baseline, the curves after falling by up to 75% of their original values recovered with time and achieved second peaks. It is not, however, always true that a curve after falling to a lower level may ascend to attain a second peak or gradually drop to the baseline. A different phenomenon was observed with *E. coli* whereby after the curve dropped to 44% of the value at treatment it remained at that level for the rest of the experimental period and started to drop after several hours (Fig. 7.4 i). The length of time a *p-t* curve needed to revive and come back to the same level as before treatment and subsequently achieve a second peak was positively

correlated with the level of the drop of the *p-t* curve (Fig. 7.9), which is in turn positively correlated with the concentration of propolis for each propolis sample (Fig. 7.8).

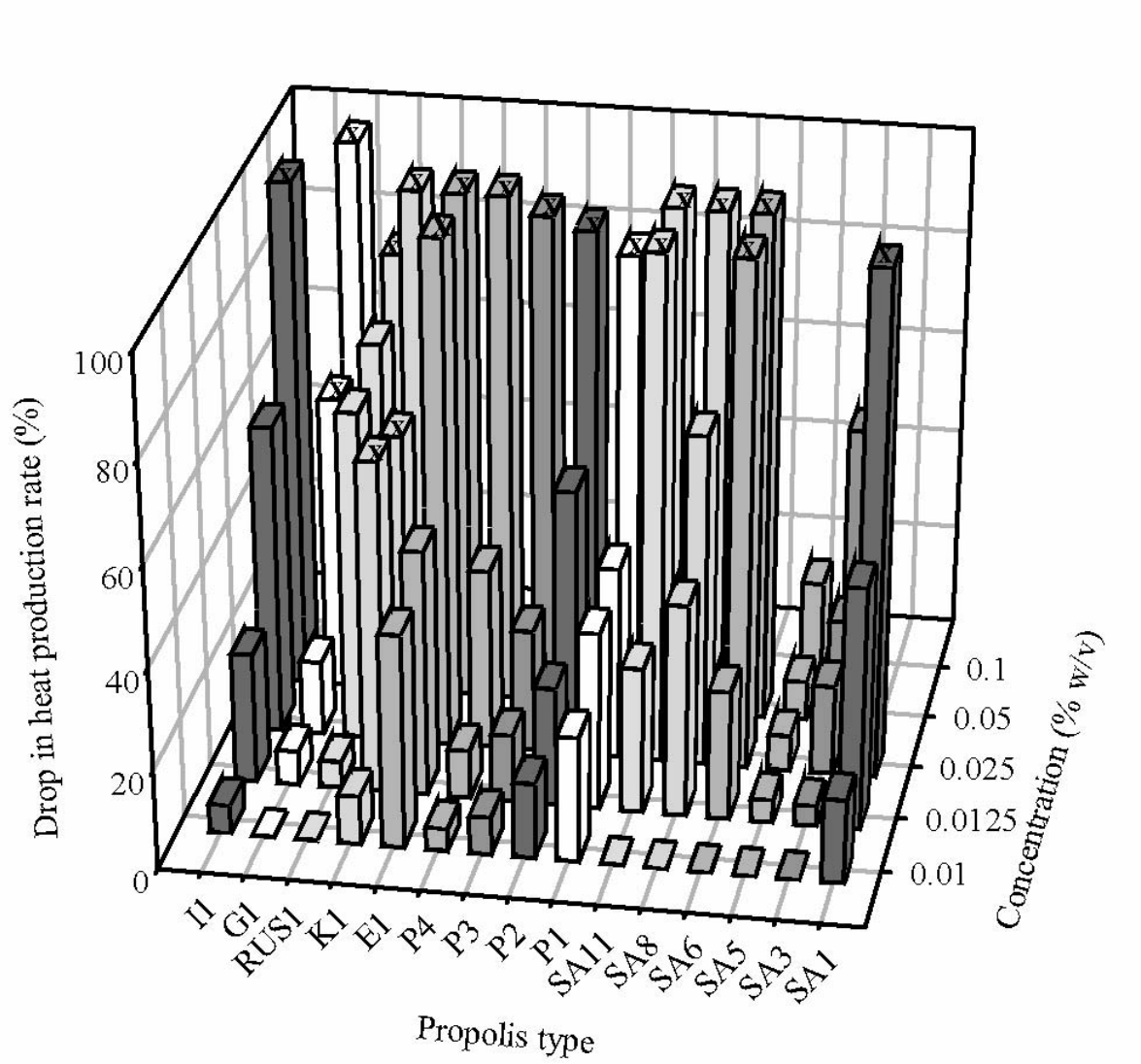


Fig. 7.8 Effect of EEP treatment on the heat production rate of *B. megaterium* demonstrated by the percentage drop in the level of the *p-t* curve. Control treatments with 60% ethanol and distilled water showed no effect. The "x"s at the top of the bars indicate bacterial death and drop of the curves to the baseline suddenly or gradually.

The relations between the level of drop of the *p-t* curve and the time needed for the curve to recover to the same level as before vary from sample to sample significantly as exemplified in Fig. 7.9. Even though significant differences between the various samples existed, all showed the same trend of ascending recovery time with the increase in the level of drop of the *p-t* curve. For this reason, the data for all propolis samples were pooled together and a correlation was made between the pooled drop in the *p-t* curve and the pooled recovery time (Fig. 7.9), and they were positively correlated with a coefficient of determination r^2 = 0.63.

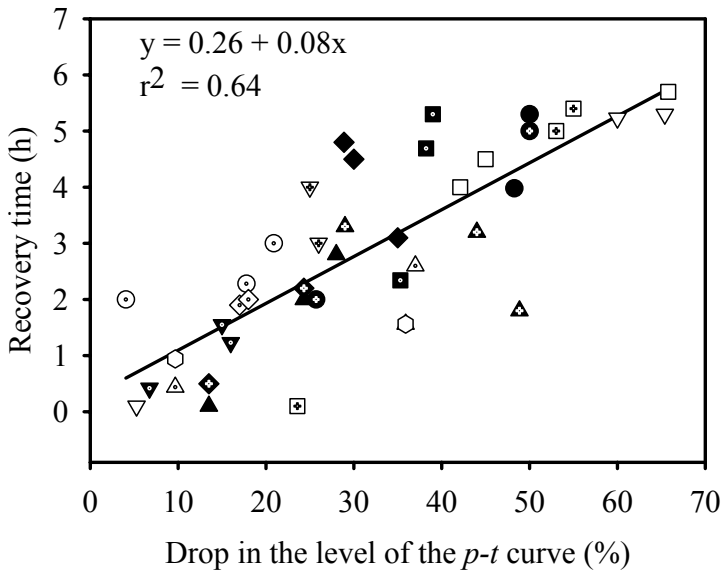


Fig. 7.9 Relation between pooled percentage drop in the level of the *p-t* curve of *B. megaterium* after treatment with 0.0125, 0.025, and 0.05% w/v EEP from different geographic origins, and the time needed for the curve to recover to the same level as before treatment. Pooled results for the 16 propolis samples investigated.

7.4.5.3.3 Slope of curve ascend after treatment

The treatment of an exponentially growing bacterial culture with propolis causes a drop of the *p-t* curve to a lower level, and the curve may ascend again to achieve an after treatment peak. The slope of ascend after treatment could be an important criterion to compare the antimicrobial activities of different concentrations of a sample or a given concentration of various samples, since it could be a reflection of the mechanism of action of the antimicrobial agents.

The treatment with propolis affected the slope of ascend of the curve of a surviving culture (Table 7.5). Actually the length of time needed for the curve to recover to the same level as before is determined by the length of the second lag phase incorporated into the curve due to treatment and the slope of ascend of the reviving curve. The steeper the slope of ascend and the smaller the second lag phase, the shorter is the curve rebound time.

In order to use the slope of ascend as a criterion to compare the effect of different samples and concentrations, the slope of ascend of the exponential phase of growth after treatment was divided through the slope of ascend of the curve before treatment at the exponential phase and multiplied with 100. For almost all propolis samples it was observed that the treatment with lower concentration influenced the slope of ascend less than the treatment with a higher concentration. It was also observed that the treatment with very weak concentrations of some propolis types promoted the growth of *P. syringae* and the curves

ascended at significantly higher rates after treatment than before. Typical examples for this phenomenon were the treatments with 0.01% K1, 0.0125 P1, 0.0125 P2, 0.0125 P4 (Table 7.5). The phenomenon of hormesis for *P. syringae* was also observed in the Petridish bioassay experiments whereby dense bacterial growth was encouraged around wells containing concentrations lower than MIC or slightly higher than MIC values. In the latter case the dense growth zone was preceded by inhibition zone around the well (Fig. 7.1, and see appendix Table A1).

Table 7.5 Effect of propolis on the calorimetric power-time curves
Effect of the treatment of an exponentially growing culture of *B. megaterium* on the subsequent features of the calorimetric *p-t* curve displayed by (i) the percentage ascend of the slope of the curve after treatment compared to that before treatment, and (ii) the percentage level of the peak after treatment compared to the level of a control peak. Since higher concentrations of K1 caused lethality, the effect of a 0.01% on the curve is shown here.

	After treatment with 0.0125% w/v EEP		After treatment with 0.025% w/v EEP	
Propolis	% Slope	% Peak	% Slope	Peak
E1	33.8	99.5	declining	none
G1	96.2	100.8	80.4	97.8
K1	138.1 (for 0.01%)	96.8 (for 0.01%)	none	none
Rus1	100.0	98.2	46.3	98.8
I1	95.5	99.7	97.4	100.3
P1	119.6	101.1	65.6	102.5
P2	187.5	103.2	58.5	102.1
P3	72.3	95.5	66.1	95.8
P4	119.4	95.8	38.9	98.4
SA1	70.4	96.1	74.3	98.2
SA3	97.7	104.0	57.3	106.3
SA5	92.6	106.1	106.3	101.5
SA6	37.7	97.5	declining	none
SA8	58.5	98.8	58.9	97.7
SA11	47.8	99.6	declining	none

7.4.5.3.4 Level of the *p-t* peak achieved after treatment

As it was not clear whether the treatments with propolis resulted in different levels of *p-t* peaks, like the different levels of drop and subsequent features of the curves, it was necessary to compare the levels at which peaks of the treated culture *p-t* curves were achieved. As the peak levels of untreated culture of *B. megaterium* were very similar to each other with a mean ± SD value of 0.447 ± 0.004 μW ml^{-1}(n = 5), comparison of individual values with the mean was considered reliable. The treatment peaks lay in the range of 95.5% to 106.3% of the level of the control peaks with a mean of 99.6%, with no significant difference (student's t-test, $p > 0.05$). No significant differences were observed among the propolis samples or concentrations (2-way ANOVA, $\alpha = 0.05$) (Table 7.5).

7.4.5.4 Comparison of the antimicrobial activities of different propolis extracts

Three different extracts, ethanol-extracted propolis (EEP), water-extracted propolis (WEP) and volatile components of propolis (PV) of the sample G1 were compared using several calorimetric curve parameters to observe if there was any difference in the kinetics of action against *B. megaterium*. The MIC and MBC values for these extracts were, respectively, 0.0125 and 0.05 for EEP, 0.05 and 0.25% w/v for PV, and 0.1 and 0.5 for WEP. EEP required less concentrated solutions to show an inhibitory action and total microbial death than PV followed by WEP. Apart from these differences, no basic difference in the pattern of kinetics of antimicrobial action could be found. The dose-response curves of concentration versus drop in the level of the *p-t* curves due to treatment with the different extracts showed the same pattern but at different concentrations (Fig. 7.10 a). Not only did they show resemblance in the level of drop of the curves but also with the slope of ascend of the reviving *p-t* curves, and the level at which the peaks were achieved after treatment (2-way ANOVA, $p > 0.05$, $n = 3$) (Fig 7.10 b).

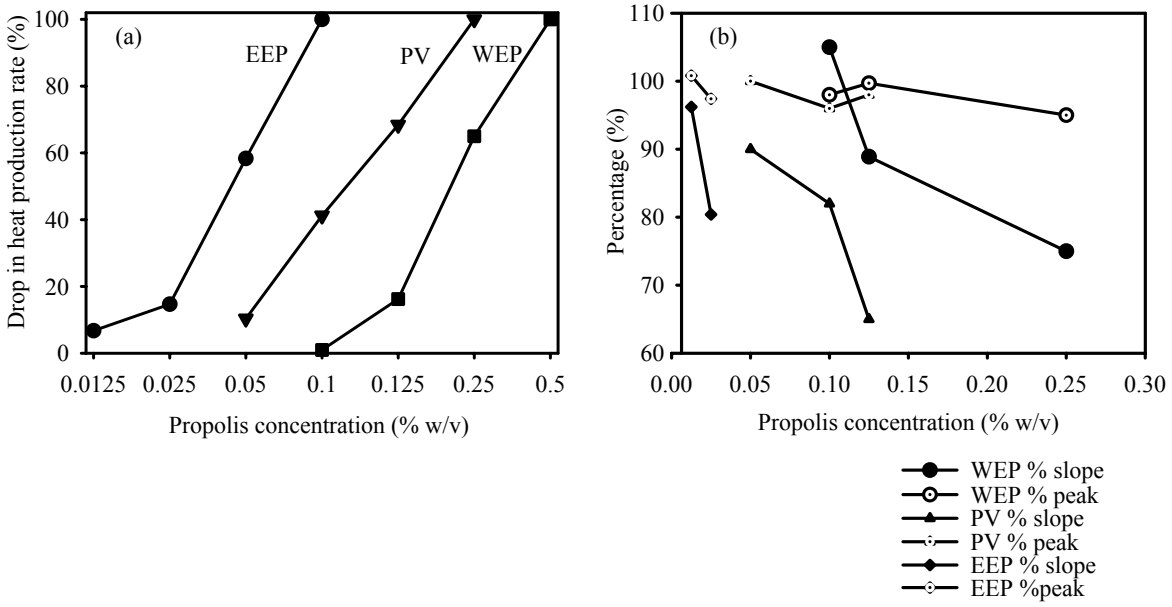


Fig. 7.10 The effect of treatment of an exponentially growing culture of *B. megaterium* with different concentrations of EEP, of PV, and of WEP on (a) the percentage drop in heat production rate after treatment compared to that before treatment, (b) the percentage ascend of slope of the curve after treatment compared to that before treatment and the percentage level of peak after treatment compared to a control peak.

7.5 Discussion

7.5.1 Physico/electro-chemical properties of propolis and yield of extraction

pH values: Unpredictable differences in pH values of the extracts of the different propolis samples exist with no relation to their geographic origin, the species or subspecies of bees that collected them, the yield of extraction, the quality of propolis, and the method of extraction. This electrochemical property of solutions is affected by the quantity and/or quality of acidic/basic substances in the solution. It is rather a reflection of the proportion of positive and negative charges that are found in a solution, and thus samples completely different in their chemical composition may have similar pH values based on the proportion of charges they possess. For this reason the changing pH values between samples even from the same apiary should not be surprising since such samples have already shown difference in the quality and/or quantity of certain chemical groups they possess. Neunaber (1995) demonstrated, using multiple samples from several countries and geographic locations, that the concentration of phenyl-substituted carboxylic acids such as benzoic acid, caffeic acid, ferulic acid, quercetin, cinnamic acid, pinocembrin, pinobanksin, galagin, etc., from the same beehive varies highly based on the year and/or the season of collection. In addition, she also mentioned that the differences between samples from the same apiary may be higher than that between samples from completely different geographic locations. Greenaway et al. (1990) observed a very high quantitative difference (eg. 2 % versus 20% caffeic acid and its esters) in the composition of propolis samples collected 400 meters apart from each other in Oxford. The researchers attributed this difference to the different clones of *Populus sp*. visited by the bees, each clone of the plant being characterized by its own specific chemical composition.

Electrical conductivity: The heterogeneous nature of electrical conductivity of similar extracts of various samples could be explained based on the quantitative and/or qualitative difference in the content of mobile charge carriers (free electrons and/or free ions) in the samples. The larger the total concentration of mobile charge carriers, the higher is the electrical conductivity. A clear difference was observed between the different extracts the strongest value for WEP, and the weakest for PV. This could be due to the fact that water extracts only the charged/polar components of propolis and hence has a higher concentration of free ions (electrolytes) compared to the other two, which possess a bulk of non-polar components with little amount of polar components. The electrolytic potential of polar components in EEP and PV is weakened due the dilution factor by the non-polar groups. Results similar to the findings in this chapter were published by Lindenfelser (1967), and König and Dustman (1988). Due to the physico-chemical heterogeneity of propolis samples

from the same location and apiary, the use of electrical conductivity as standardization criteria was put under question by König and Dustmann (1988) and is confirmed here too.

Types of extracts: The extraction of propolis with ethanol helps to procure, among others, flavonoids and phenyl substituted carboxylic acids (Neunaber 1995), of which the flavonoids make up about 95% of the total (König and Dustmann 1988); but wax and fat remain unextracted (Woisky and Salatino 1998). Phenolics (flavonoids and phenyl substituted carboxylic acids) are ubiquitous plant secondary metabolites and hence are found in all propolis samples (König and Dustmann 1988, Greenaway et al. 1990, Burdock 1998). They make up more than 50% of the total weight of raw propolis (Bankova et al. 1994).

Ethanol (70%) extracts those components soluble in alcohol and water. In addition to that it may also extract the volatile components of propolis. Water extracts only the polar components of propolis such as phenyl substituted carboxylic acids and their esters (e.g. cinnamic acid and caffeic acid that make up to 44% of the total water soluble components of propolis, Neunaber 1995). Other polar components that can be extracted with water include amino acids, and sugars (Marcucci 1995, Nagai et al. 2003). Intuitively it can be deduced that the water soluble components of propolis make up a small fraction of the total extractable components of propolis, as demonstrated in the present investigations too. The lower proportion of the water extractable components was also demonstrated by other researchers ranging from 2.6 to 6.3 w/w (Spiridonov et al. 1992, Neunaber 1995), 2 to 4% for Chinese propolis and 6 to 14% for Brazilian propolis (Miyataka et al. 1997).

The volatile components of propolis (essential oils) consist mainly of terpens and terpenoids (mono-, sesqui-, di-, tri-). Other constituents of volatile oils include alcohols, mainly aromatic alcohols, phenols, aldehydes, ketones, acids (from acetic to stearic acid), esters, a series of alkanes, alkylated benzenes and naphthalene (Bankova et al. 1994). The variation in the volatile oil composition of propolis from different locations is higher than in their phenolic components (Bankova et al. 1994). Since the extraction with water involves heating of the setup, the water soluble components of propolis may also possess the propolis volatiles, but the extraction of PV extracts only the volatile oil components of propolis. For this reason the yield of extraction of propolis is in the order of PV < WEP < EEP. The volatile oil components of propolis were shown to make up 0.2 to 1.28% (Bonvehi and Coll 2000), 0.28 to 0.49% (Woisky and Salatino 1998), 0.27 to 0.60% (Bankova et al. 1994), 0.1 to 0.6% w/w (Kujumjiev et al. 1999) for propolis from different geographic samples. These results are similar to the yield of volatile constituents of propolis displayed in Table 7.1.

The difference in the yield of extraction between different propolis samples is to be expected as it could be affected by the resin content of the material the bees collect from the plant and the amount of wax they mix with it. The resin and wax content of propolis could differ based on geographic location, vegetation composition, bee species and availability of plants (Meyer 1956, Johnson et al. 1994). In seasons and locations where propolis source plants are scarce, the colony suffers propolis shortage and bees were observed collecting "propolis substituents" like asphalt, paint, mineral oils (König 1985). These propolis substituents are then mixed with the available propolis resin and used in the beehive (König 1985). In addition to the non-propolis materials collected from the environment and mixed with propolis, the bees can also control and vary the proportion of wax they add to the plant resin based on the availability of plant resin and the purpose for which propolis is to be used (Burdock 1998). For example propolis used to repair honeycombs is often supplemented with large quantities of wax to give it a firmer composition, while propolis applied in a thin coat to the surface of a comb usually contains little or no wax (Meyer 1956). Thus the lower yield of some propolis samples that looked pure up on visual inspection could be contributed to the higher wax content which can not be extracted in 70% ethanol.

The lower yield of "impure propolis" samples is due to, among other factors, the higher proportion of foreign materials that the bees collect and use as propolis substituents, and also substances incorporated to propolis by the beekeeper during the collection of propolis. The quality of propolis is dependent on how propolis is procured from the frames, cover boards, collection matt, and the inner wall of the beehive (Bogdanov 1999). The stingless bee, *Tetragonisca angustula* Illger is normally known to collect propolis and mix it with wax and soil to form geopropolis (Bankova et al. 2000). Thus soil is the normal component of geopropolis from stingless bees imparting lower yield to this type of propolis. A comparative investigation on the chemical make-up of propolis from stingless bees and honeybees from the same geographical location showed that the two propolis samples have similar yield and also resemble in chemical composition with slight differences that could be traced to the preferred trees visited by the two bee species (Pereira et al. 2003).

7.5.2 Petridish bioassay of different propolis samples

The results of antimicrobial tests are unambiguous proofs, that in spite of the great difference in the chemical composition of propolis of different geographical origins and collected by various bee races, all of them exhibit significant antibacterial and antifungal effects. But the strength of antimicrobial activity could differ based on the nature of the

specific substances in each sample. The differences in the strength of antimicrobial action between the various propolis samples may not be surprising since the composition and proportion of the bioactive components in the different samples differ from each other quantitatively and/or qualitatively. Kedzia et al. (1990) proposed that the antimicrobial action of propolis is complicated and could be due to the synergism between flavonoids, hydroxyl acids, and sesquiterpenes. It was experimentally demonstrated that not even a single component isolated from propolis showed an activity higher than the total extract (Metzner et al. 1977, Kujumgiev et al. 1993, Bonvehi et al. 1994). The synergistic effect between the different components of propolis was already reported by Scheller et al. (1977), and latter confirmed by Krol et al. (1993). It is thus obvious that, in different samples, different substance combinations are essential for the biological activity of propolis, rather than only one, and hence samples of completely different geographic origins may have comparable antimicrobial activities (Kujumgiev et al. 1999).

Though no chemical analysis was performed to identify the bioactive components in the samples used in the present investigations, it is possible, based on literature data of propolis from different geographic origins, to state that the samples do differ in their chemical composition. The degree of variation depends on the type of plants visited by the bees, which is in turn determined by the geographical location (for example Ghisalberti 1979, Marcucci 1995, Miyataka et al. 1997, Burdock 1998, Kujumgiev et al. 1999, Bankova et al. 2000, Bankova et al. 2002). Though the specific chemical make-up of the plant resins collected by the bees differs based on the plant species visited, the general framework of bioactivity is more or less the same. Regardless of the geographic location, where a plant grows, the purposes for which it secretes resin resemble at least partially. Resin is mainly secreted by plants in order to seal wound, to stop sap loss and protect wounds from infection by microbes, to protect against infection of pollen (it is coated with resin), to stop germination of seeds and sprouting of bud while frost (Ogren 1990). Though the specific chemicals that are responsible for these actions could differ, the essence of action remains the same, leading to the similar biological activity of different samples.

7.5.3. Comparison of the sensitivity of different microbes to propolis

All of the Gram positive bacteria tested were highly sensitive already to lower concentrations of propolis, but the Gram negative bacterium *E. coli* displayed a reduced sensitivity to most of the samples and was insensitive to 2 of the 16 samples tested. The lower sensitivity of *E. coli* is in agreement with findings by several researchers where this bacterium

showed either very low sensitivity or total insensitivity against propolis (Digrak et al. 1995, Marcucci 1995, Kujumjiev et al. 1999, Bonvehi and Coll 2000, Sforcin et al. 2000). It can not, however, be generalized that Gram negative bacteria are insensitive to propolis since the other Gram negative, *P. syringae*, even though it showed relatively higher MIC values, had a sensitivity similar to most Gram positive bacteria at higher concentrations of propolis. But it can be ascertained that the Gram negative bacteria are less sensitive than the Gram positive ones, at least at lower concentrations of propolis, which is in agreement with literature data (Grange and Davy 1990, Dobrowolski et al. 1991, Sforcin et al. 2000). The most plausible explanation for the less sensitivity of Gram negative bacteria is their outer membrane that inhibits and/or retards the penetration of propolis at lower concentrations. After a certain threshold concentration, however, the impermeability of this membrane is disturbed resulting in the movement of bioactive components of propolis into the cell interior, resulting in cell inhibition or death. Another possible reason why the Gram negative bacteria are more resistant to propolis might be the possession of multidrug resistance pumps (MDRs), which extrude amphipathic toxins across the outer membrane (Tegos et al. 2002). The presence of MDRs in *E. coli* and their role in the insensitivity of the bacterium to antimicrobials was clearly elucidated (Lomovskaya and Lewis 1992, Nikaido 1999, Zgurskaya and Nikaido 1999, Lewis and Lomovskaya 2001). The MDRs could be very effective at lower concentration of propolis and extrude those molecules that crossed the outer membrane barrier. This pumping potential of the MDRs can be saturated at a certain threshold concentration and the rate of penetration and accumulation of antimicrobials in the cell interior increases, resulting in antimicrobial effect at higher concentrations.

Fungi are generally less sensitive than bacteria in terms of the MIC values and/or diameter of inhibition zones at higher concentrations, except for the yeast that showed higher diameters like that of the bacteria. Considering the MIC values, the yeast had a sensitivity in between the highly sensitive bacteria and the less sensitive mould.

The saturation in the dose response curves for the filamentous fungi, with little change in the diameter of the inhibition zone as the concentration increases could be due to the weak solubility/insolubility of propolis in the agar layer. The weak diffusion potential of propolis in agar layer and hence unexpectedly small diameters of inhibition zones were observed by other researchers too, when using higher concentrations of propolis against fungi (Metzner et al. 1979, Sawaya et al. 2002). The strongly non-polar components of propolis precipitate on the inner wall of the agar hole, or after diffusing a very short distance, blocking the diffusion path and retarding migration of potentially diffusible substances. So, for the diffusion of propolis

at higher concentrations, the general principle of diffusion, that rate of diffusion increases with increasing concentration may not work. After the end of the experiments, all Petridishes containing higher concentrations of propolis such as 4%, 8% and 10% displayed propolis precipitations at the inner wall and on the bottom of the hole.

The presence of certain bacteriostatic/bactericidal chemicals in the growth medium at a concentration lower than a critical inhibitory level could enhance the growth of an organism that otherwise would have been inhibited/killed by higher concentrations, a phenomenon known as hormesis (Edward et al. 1998). The phenomenon of hormesis was displayed by *P. syringae* at concentrations of propolis lower than MIC for most propolis samples. This situation alerts that if propolis is to be used in treatment of infections it has to be used at concentrations far above the MIC values, in order to minimize the risk of encouraging bacterial growth at or in the immediate surrounding of the site of application.

7.5.4 Comparison of the antimicrobial activities of different propolis extracts

EEP showed the highest antimicrobial activity compared to the other two extracts, even though the differences between EEP and PV were not significant for most samples. The reason why EEP is superior to WEP, and in some case to PV is that extraction of EEP extracts all water and ethanol extractable and biologically active components that are also present in the other two extracts. In addition, EEP contains several bioactive components that are not found in WEP and PV. Since it is already pointed out that the antimicrobial activity of propolis could involve a synergistic interaction between its components (Scheller et al. 1977, Kedzia et al. 1990, Krol et al. 1993, Kujumgiev et al. 1999) the possession of higher activity by EEP would not be surprising. Regardless of the possession of few types of biologically active compounds (Kujumgiev et al. 1999) the volatile components, astonishingly showed activities comparable to EEP. This could be due to the fact that even if PV contains fewer types of compounds compared to EEP, it may contain a sufficiently high amount of bioactive compounds to result in an effect comparable to the former. It is known that the PVs possess good to moderate antibacterial activities and they are responsible for the lower incidence of microbial aeroflora within the apiary (Ghisalberti 1979, Petri et al. 1986). Since the rest of propolis is insoluble in the beehive interior and hence “inactive”, the volatile components of propolis are essential to reduce infections in the bee hive. The yield of extraction and consequently the antimicrobial action of the volatile components depend on the age and storage situation of propolis (Woisky and Salatino 1998). If it is not stored in a tight container, components are lost with time and the sample loses its potential. This may be the

reason, apart from the very nature of chemical make-up of the various propolis samples, for the different yield and antimicrobial activities of the PVs from various samples investigated in this study.

7.5.5 Calorimetric experiments

The results of the Petridish bioassay experiments were affected by the insolubility of propolis in the agar layer, especially while using highly concentrated propolis against the relatively insensitive fungi. The insolubility problem was not serious at lower propolis concentrations tested against bacteria since the highly diluted hydro-insoluble components could diffuse through the agar layer with the excess solvent in which they are dissolved (ethanol). The problem of insolubility could still be there at lower concentrations and affect the diffusion potential, though not clearly observable. The calorimetric results, however, were not and can not be affected by this problem since it is done in a nutrient broth. Due to vigorous stirring of the culture, the water insoluble components of propolis remain suspended in the medium and express their antibacterial activity.

One of the limitations confronted with calorimetric experiments was that the calorimetric spiral, where heat production is detected, and the fermenter where cultivation of cells is done, are separated by an unavoidable geometric distance. This distance is necessary since a minimum tube length is required for the precise thermal equilibration of the culture before it arrives in the calorimetric spiral. The culture in the fermenter is aerated continuously, and as a result, the bacterial growth is not limited by oxygen. Growth of the bacterial culture thus continues until carbon and energy supplies in the fermenter are exhausted, marked by a declining phase of count of CFU. The situation in the tubing system and hence the calorimetric setup is, however, different. As a culture sample leaves the fermenter and enters the tubing system it possesses a certain concentration of oxygen, which is continuously consumed and reduced by the metabolizing bacteria. At a low cell density, bacterial growth and metabolism continue, since there is sufficient oxygen for the bacteria to utilize, even though the concentration of oxygen decreases continuously. But, this situation changes at higher cell densities. The available and ‘in the flow line non-replenishable’ oxygen is consumed nearly totally before the culture arrives at the calorimetric spiral, and hence bacterial metabolism and heat production rate drop drastically in the tubing system. In case of the facultative anaerobe, *E. coli*, a different situation was observed. Upon exhaustion of oxygen in the flow line the metabolic rate started to drop until it reverted and ascended again because of fermentation. Fermentation was initiated after the online oxygen concentration

dropped to about 30 μmol l^{-1}. The anaerobic curve ascended further and achieved a plateau level at about 0.5 μW ml^{-1} and remained at this level for the rest of the experimental period until it started dropping due to complete exhaustion of nutrients (carbon, nitrogen and energy sources). The constant level of heat production rate indicates that there is no significant bacterial growth taking place, and that the heat output is mainly due to maintenance metabolism, not of growth. This fact is also demonstrated by the nearly constant level of CFU in the anaerobic phase.

Due to depletion of oxygen in the flow line the declining calorimetric curve may not be a good indicator of the metabolic activity in the fermenter itself after a growth peak is achieved in the calorimeter (Hölzel et al. 1994, Garedew and Lamprecht 1997, Garedew et al. 2003). The calorimetric death phase may actually be the late exponential as well as the stationary phase of bacterial growth in the fermenter. Therefore, killing some bacteria in the fermenter and the flow line by applying a moderate concentration of propolis may open an opportunity for the survivors to utilize the excess oxygen and nutrients available, thrive and increase the heat production rate showing the true picture of the phenomena that take place in the fermenter. The treatment at this phase could also help to find out whether the drop in the heat production rate was caused only by the shortage of oxygen in the flow line or due to exhaustion of nutrients in the fermenter and flow system as a whole. If the drop of the heat production rate was caused by exhaustion of nutrients, the treatment with propolis should not result in a boost of the heat production rate. But the treatment with a very strong concentration of propolis may kill the bacterial culture in the fermenter and no heat production could be expected. For this reason this treatment was done only with moderate concentrations of propolis.

Therefore, due to the above mentioned problems associated with length of the tubing system and subsequent oxygen depletion, calorimetric recording of the heat production rate is a true picture of events taking place in the fermenter only at lower cell densities. If calorimetric data are to be used at higher cell densities, the results have to be compared with other data, such as the oxygen concentration in the flow line and the number of CFU, and results have to be interpreted with caution.

It has been well documented that the sensitivity of bacteria to antimicrobials is highly affected, among other factors, by the growth phase at which treatment is done (Hogan and Kolter 2002). Slow growth rates of bacteria resulting from nutrient limitations are accompanied by an increased resistance to multiple antimicrobial compounds because of low metabolic activities and decreased cell permeability (Brown and Williams 1985).

Furthermore, slow growth is also correlated with the increased expression of multi-drug efflux genes (Rand et al. 2002). A recent research finding showed that *E. coli* stationary phase cultures produce a diffusible factor that induces resistance to antibiotics in growing *E. coli* cells but the nature of the signal and the response of the cells is not yet clear (Heal and Parsons 2002). It has also been proposed that, in stationary phase cultures, there are subpopulations of antibiotic resistant 'persister cells' that lead to the increased resistance to antibiotics (Spoering and Lewis 2001). Persister cells are not mutants and do not produce antibiotic resistant offspring in the absence of antibiotics. It is not yet known how persister cells are formed or what factors contribute to their antibiotic resistance (Hogan and Kolter 2002). Due to these facts, treatments of the bacterial cultures were done at the exponential growth phase, before the calorimetric *p-t* peaks were reached.

The treatment of bacteria with propolis in the calorimetric exponential phase resulted in a drop of the heat production rate to a lower level, depending on the concentration of propolis. If the concentration is weak, it does not kill all bacteria, and hence the survivors do continue to metabolise, maintaining the heat production rate at a certain level above the baseline. This level is kept for some time directly depending on the concentration of propolis, after which the curve revives and ascends again. The most plausible explanation for this behaviour could be that, after treatment a certain proportion of the cells is killed, others are inhibited, and some others remain unaffected. The metabolic heat production rate in this case could originate from the inhibited cells, since they could perform maintenance metabolism, and those of the unaffected and normally metabolizing and growing cells. It may be possible that the number of normally metabolizing cells is very low and the change in heat production rate is below the detection limit of the calorimeter. But with the course of time, the number increases and the change in their metabolic rate could be detected on the *p-t* curve. The survivors then metabolise and grow in the flow calorimeter displaying the *p-t* curve typical for the bacterial strain. Another possible explanation might be that some bacteria could be inhibited and perform their maintenance metabolism for a certain period of time. The inhibition may then come to end, and the bacteria start metabolizing and growing again displaying features typical of a normally growing bacterial culture. The second hypothesis agrees with the proposal by Maillar (2002) that bacteriostatic effects achieved by lower concentration of biocides might correspond to a reversible activity on the cytoplasmic membrane and/or impairment of enzyme activity.

The treatment of bacteria with EEP dropped the heat production rate and raised the online and fermenter oxygen concentration immediately, but the drop in the number of CFU

was gradual. A plausible explanation for this could be that immediately after treatment, the metabolic rate and oxygen consumption drop drastically even though the organisms were not dead, but weakened. Removal of a sample and culturing it on propolis free medium releases the bacteria from the antimicrobial agent and they start growing. But with the increase of incubation time with propolis in the fermenter the number of dying bacteria is increasing and hence the CFU curve declines. The results of the present investigation are in agreement with that of Sforcin et al. (2000), whereby incubation of a sensitive *S. aureus* strain and a relatively less sensitive *E. coli* one in broth containing EEP, and subsequent removal and plating of the samples displayed different patterns of incubation time versus survival curve, with the sensitive bacteria dying in short incubation time.

Since the *p-t* peaks after treatment were achieved at the same level of heat production rate and oxygen concentration in the flow line, as for the control culture, it can be ascertained that the treatment with propolis did not affect the level of oxygen sensitivity of the survivor bacteria. The lower slope of ascend of the *p-t* curve after the treatment with a higher concentration of propolis could be an indication of partial inhibition of cell division of the survivors thus growing and metabolizing at slower rates. The effect of propolis on cell division has already been confirmed (Takaisi-Kikuni and Schilcher 1994).

The calorimetric method displayed MIC values lower than the Petridish bioassay method. The main reason for this could be that the calorimetric method is an online recording of activity and hence even the slightest and short lasting effects could be detected. But in case of the Petridish bioassay method results are cumulative effects of incubation for 24 to 48 h. In addition to that, several other problems encountered by the Petridish bioassay method do not affect the calorimetric results. Results from the Petridish assay method are influenced by inoculum size, agar layer consistency, incubation temperature, polarity and diffusion potential of the test substance. At a first glance, it seems that the inoculum size affects the calorimetric results too, but since treatment can be done at a level of the exponential phase of the *p-t* curve, which corresponds to an exactly predetermined density of CFU, this plays no role. Nevertheless the size of the inoculum affects the length of the lag phase, which has nothing to do with the treatment at the exponential growth phase.

The phenomena of change in the structure of the curves after treatment at the calorimetric death phase could be attributed to the shortage of oxygen in the flow line that resulted in a drop of the *p-t* curve to the minimal point after the peak. The treatments with propolis inhibit/kill some cells in the fermenter and flow line, increasing the availability of oxygen for the survivors, which results in an increased metabolism and ascend of the curve.

7.5.6 Possible mechanisms of propolis action

Apart from the proposal and speculation by several researchers as to the mechanism of action of propolis, no much work has been done, and literature data are scarce. It was pointed out that the mechanism of action of propolis is complex and no analogy can be made to any classic antibiotics (Kedzia et al. 1990).

The slow growth rate of bacteria and the lower slope of ascend of the *p-t* curve after treatment with a moderately concentrated propolis solution is an indication that the rate of growth/cell division and metabolic activity of the survivors were partially inhibited or reduced. Electron microscopic investigation of propolis treated bacterial cells by Takaisi-Kikuni and Schilcher (1994) demonstrated that bacterial cell division was inhibited and they argued that the mechanism of action could be like that of nalidixic acid which is known to inhibit DNA replication and subsequent cell division. The researchers also argued that propolis could inhibit DNA dependent RNA polymerase. It was also displayed that treatment with propolis interferes with protein synthesis, proved by measuring the concentration of extracellular proteins of control and treated cells (Simuth et al.1986, Takaisi-Kikuni and Schilcher 1994)

The relatively lower sensitivity of Gram negative bacteria compared to the more sensitive Gram positive ones, at least at lower concentrations, could be traced to an activity that involves the Gram positive bacterial cell wall, inhibition of cell wall synthesis and hence distortion of its integrity. Electron microscopic pictures displayed that propolis treated cells possessed defective cell walls and failed to separate after cell division and formed a pseudo-multicellular structure (Takaisi-Kikuni and Schilcher 1994). In an experiment with a known antibiotic, Nishino et al. (1987) demonstrated that the formation of a pseudo-multicellular structure after treatment could be due to the blockage of the so called splitting system of the cross wall. Treatment of *S. aureus* with a combination of benzylpenicillin and propolis showed a synergistic effect witnessing that the effect of propolis could involve inhibition of cell wall synthesis (Schub et al. 1981).

Electron microscopic pictures of treated cells displayed that the cytoplasm of the cells was disorganized and it was emptied after a prolonged incubation time (Takaisi-Kikuni and Schilcher 1994).

Antifungal activities of propolis are supposed to be like that of amphothericin B, which forms complexes with sterols (ergestrol) of the fungal membrane (Cizmarik and Trupl 1975, Pepeljnjack et al. 1982).

It can be concluded that the present results are unambiguous proofs that in spite of the enormous differences in the chemical composition of propolis from different geographic locations, all samples exhibited significant antibacterial and antifungal activities. Hitherto investigations dealing with chemical composition and biological action of propolis did not point out one individual substance or a particular substance class which could be responsible for this action. Obviously a synergistic interaction between different substance combinations is essential in different samples for the biological activity of bee glue. It seems that the chemical nature of propolis is beneficial not only to bees but have general pharmacological values as an antimicrobial natural product.