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Microcalorimetric investigation on the antimicrobial activity of honey of the stingless bee *Trigona* spp. and comparison of some parameters with those obtained with standard methods

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Abstract

The ever-increasing report of drug resistance by bacteria and side effects of certain pharmaceutical products are leading to aversion to some synthetic drugs and resurgence of the use of alternative therapies, such as apitherapy i.e. therapy with bee products. Honey of the stingless bee, *Trigona* spp., is used in Ethiopia as a panacea against dozens of ailments and considered to be superior to honeybee honey.

The kinetics of antibacterial actions of two stingless bee honey samples obtained from Ethiopia were investigated using a flow calorimetric method. Four Gram-positive and two Gram-negative bacterial species were tested in vitro. The minimal inhibitory concentration (MIC) values from the calorimetric experiments were compared with those obtained from petridish and spectrophotometric methods.

The calorimetric method displayed antimicrobial activities of weak honey concentrations that could not be detected with standard microbiological methods. Calorimetric results also indicated that lower concentrations of honey have bacteriostatic where as higher concentrations show bactericidal actions. The MIC values obtained from the calorimetric method were several folds lower than those from the petridish and spectrophotometric methods, showing the very high sensitivity of the former one.

Both honey samples have broad-spectrum antimicrobial actions against both Gram-positive and Gram-negative bacteria.

Keywords: Stingless bee honey; *Trigona* spp.; Calorimetry; Bactericidal; Bacteriostatic; MIC

1. Introduction

Honey has been used in medicine against bacterial infections for thousands of years. It is however replaced by antibiotics in modern times [1]. The continued use of some systemic or topical antimicrobial agents has imparted the selective pressure and led to the emergence of antibiotic resistant strains [2]. The ever-increasing reports of drug resistance by bacteria and of side effects of certain pharmaceutical products are causing an aversion to and subsequent cease of the use of some synthetic drugs and antibiotics. To solve such problems researchers are on an unremitting search and synthesis of new drugs.

Unfortunately, the costs of some of such drugs are soaring and the rate of discovery is becoming low [3]. These phenomena are leading to resurgence of interest in the use of alternative therapies such as apitherapy (therapy with bee products), among several others, and the general "back to nature" trend in solving regional problems with indigenous knowledge. Of the several products of the beehive, the most commonly used food/health supplement with a resurrecting use in medicine is honey [4,5].

The honey broadly applied for different purposes is mainly obtained from the western honeybee *Apis mellifera*. In the tropical and subtropical regions, however, honey is made not only by the aforementioned bee species but also by several species of stingless bees that belong to the subfamily Meliponinae and the genera *Melipona* and *Trigona*. Honey of the stingless bee, *Trigona* spp., is usually a highly praised apitherapeutic agent used as a panacea against dozens of ailments in Ethiopia. Among the most common uses of stingless bee honey are to treat stomach disturbance, cough, tonsillitis, sore throat, stomach, and intestinal ulcers, cold, disease of the mouth, mucus membrane, and as

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a wound dressing. The purpose of our present investigation was to elucidate calorimetrically the mechanism of action and determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the stingless bee honey obtained from Ethiopia. In addition to that, calorimetric results will be compared with those obtained by petridish bioassay and spectrophotometric methods of our previous investigation on the same honey [6].

Nowadays, several techniques are employed in the screening of new antimicrobial agents in different laboratories. But most of the methods are associated with drawbacks and there is no standard way of presenting the results obtained by different methods [7]. Few of the most common problems with the techniques employed today are (i) inoculum size: affecting results in both liquid and solid media directly, but to be minimized by standardizing the inoculum density which is laborious and time consuming; (ii) consistency of the agar layer/activity of water affects the 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 substance: testing nonpolar substances with the petridish bioassay method is not as such credible, since the substance could not diffuse properly through the polar agar layer and hence activity could be highly restrained and; (v) color of the substance to be tested interferes with results from the spectrophotometric method, it could however be alleviated to a certain extent by using corresponding blanks for each dilution.

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. One of our aims is, therefore, to use calorimetry in the investigation of the mechanism of action of antimicrobial agents and evaluate its credibility as compared to the often-used standard microbiological methods in testing the effects of antimicrobials.

2. Materials and methods

2.1. Honey source

The two stingless bee honeys employed in our investigations were obtained from two regions in Ethiopia, where they are used as multifaceted folk medicine. Unlike honeybee honeys that are sold on every market, the stingless bee honeys are rare and can be bought only in certain regions from experts that are involved in collecting such honey. Our honey samples were: (i) Honey B (HB) obtained from Bahir Dar (11°35′N and 37°28′E) northwest Ethiopia, at an altitude of 1830 m above sea level, (ii) Honey T from Temben (13°53′N and 39°53′E) northern Ethiopia, at an altitude of 1500 m above sea level. The vegetation in both regions is categorized as Ethiopian undifferentiated woodland [8].

2.2. Bacterial strains and culture media

Bioassays of the antibacterial activities of the two honey samples were performed with: (i) four species of Gram-positive bacteria: *Bacillus subtilis* (DSM 347), *Micrococcus luteus* (DSM 348), *Bacillus megaterium* (DSM 90), *Bacillus brevis* (DSM 5609); and (ii) two species of Gram-negative bacteria: *Escherichia coli* (DSM 31) and *Pseudomonas syringae* (DSM 5176). All bacterial cultures were cultivated on Standard I nutrient broth (Merck, Lot VL 630582) and/or Standard I nutrient agar (Merck, Lot VL 694681). Media were sterilized by autoclaving at 121 °C for 15 min. The bacterial strains were bought from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany).

2.3. Calorimetric experiments

Calorimetric experiments were performed by means of a flow calorimeter of vessel volume 0.587 ml (type 10700-1, LKB, Bromma, Sweden) at a temperature of 30 °C. The calorimeter was connected by a Teflon tube of $\varnothing = 0.1$ cm to an external fermenter, a 50 ml reaction vessel with 20 ml nutrient broth. The fermenter was placed in a water bath at a temperature of 30 °C. The bacterial culture was circulated from the fermenter to the calorimeter and back to the fermenter by a peristaltic pump (LKB Pharmacia, Bromma, Sweden) at the outlet of the calorimeter in a sucking mode. Settling of cells and depletion of oxygen in the fermenter was avoided/minimized by vigorously stirring the culture with a magnetic stirrer.

To satisfy the contradicting requirements for a high flow rate (to avoid settling of cells and exhaustion of substrates during the residence time in the tubing system) and a slow one (to allow sufficient temperature equilibration, to curtail heat generation by friction and avoid reduced sensitivity of the calorimeter), a pumping rate of 56 ml h^{-1} was chosen for investigations with a prevailing aerobic metabolism, at least in the first few hours of bacterial metabolism and growth. Although it was not the main motto of our present research, but as the calorimetric sensitivity and hence the experimental results could be affected, the effect of pumping rate on the sensitivity of the calorimeter was determined by electrical calibration of the calorimeter at different pumping rates with phosphate puffer of pH 7.0.

The flow calorimetric line and the calorimetric vessel were sterilized by circulating a solution 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 setup was cleaned with potassium-phosphate buffer of pH 7.0 for 1 h.

After the flow-line was properly sterilized and cleaned with the buffer, the growth medium (Standard I nutrient

broth, Merck, Lot VL 630582) was circulated for at least 30 min to establish a stable base line. A 200 μl inoculum of an overnight Standard I nutrient broth culture was then inoculated and allowed to grow. All calorimetric experiments were performed with the four species of Gram-positive and two species of Gram-negative bacteria mentioned in Section 2.2.

Treatment of the bacteria was done at the mid-late logarithmic phase of bacterial growth by adding 2200, 1100, 400, and 200 μl of honey into the 20 ml bacterial culture to achieve a final honey dilution of 1:10, 1:20, 1:50, and 1:100, respectively. In addition to the treatments at the exponential growth phase, some experiments were also run by adding honey of different dilutions into the fermenter after the calorimetric curve has dropped to the "calorimetric death phase."

The concentration of oxygen in the flow-line and the number of colony forming units (CFU) were monitored parallel to the calorimetric experiments for selected bacterial species. Measurement of oxygen concentration was done by incorporating an oxygen electrode (WTW Cellox 325 connected to WTW Multi 340i Datalogger, Weilheim, Germany) in the flow-line at the sample outlet of the calorimeter. As the sterilizing chemicals were too aggressive to be used with the oxygen electrode, the electrode had to be sterilized separately with only 70% ethanol. Due to the labor intensiveness of the procedure of dismantling the setup, sterilizing separately and assembling the flow-line with the oxygen electrode incorporated, this experiment was done only for *M. luteus*, a representative obligate aerobe, and for *E. coli*, a facultative anaerobe, which showed a unique curve. In addition to its labor intensiveness, the chance of contaminating the sterile flow-line is also very high.

The number of CFU was determined by removing 50 μl of culture from the outlet of the calorimeter (inlet of the fermenter) at intervals of half an hour. The samples were serially diluted and plated on Standard I nutrient agar, grown for 24 h and the number of CFU was counted.

The control experiments for each honey dilution were performed by adding a volume of distilled water that equals the corresponding volume of honey, to elucidate the drop in the heat production rate due to a "dilution effect" of the liquid added rather than the antimicrobial activity.

The calorimetric signals were amplified (1000×) and recorded as power–time (*p–t*) curves by a two-channel recorder (BD5, Kipp, and Zonen).

3. Results

The sensitivity of the calorimeter is negatively affected by the pumping rate and decreases with an increasing rate. At a pumping rate of 1.5 ml h^{-1}, the calorimeter had a sensitivity of 70 μV mW^{-1}. The pumping rate used in our experiments (56 ml h^{-1}) was associated with a calorimetric sensitivity of 61.6 μV mW^{-1}.

Due to the multitude of *p–t* curves from the different treatments only representative curves of few bacterial strains will be dealt with.

Treatments of *B. megaterium* with a 1:10 dilution of Honey B in the calorimetric exponential growth phase resulted in a total mortality of cells and hence in a drop of the *p–t* curve to the baseline. In contrast, the treatment with the same dilution of Honey T reduced the heat production rate by 40% from 0.42 to 0.25 μW ml^{-1} (Fig. 1a and b). Treatment of this bacterium with a 1:20 dilution of HB dropped the *p–t* curve from 0.40 to 0.24 μW ml^{-1}, which afterwards ascended slowly and achieved a plateau at 0.34 μW ml^{-1}. But the treatment with 1:20 diluted HT brought the heat production rate down from 0.31 to 0.24 μW ml^{-1}, followed by a quick ascend of the *p–t* curve to achieve a broad peak at 0.36 μW ml^{-1} accompanied by a slow drop of the curve with incubation time. The treatment with 1:50 dilution of HB resulted in a drop of the *p–t* curve from 0.48 to 0.36 μW ml^{-1} (Fig. 1a–3). This level was maintained for nearly 1 h and subsequently ascended to achieve a peak at 0.41 μW ml^{-1}, lower than the control peak. However, the treatment with a 1:50 dilution of HT (Fig. 1b–3) reduced the heat production rate from 0.41 to 0.37 μW ml^{-1}, which rebounded immediately with a slope similar to that before treatment (0.21 μW ml^{-1} h^{-1}). As exemplified in Fig. 1 if a peak or plateau is achieved after treatment with a weak concentration of honey, it is always lower than the level of the control peak.

As seen in almost all treatments and represented by Fig. 1, the treatment with a given dilution of honey caused an immediate drop of the *p–t* curve lower than the level that is achieved some minutes later. This is because $20 - X$ ml of the total medium is in the fermenter and X ml circulating in the flow-line. Thus, the added honey is diluted at the beginning only in the $20 - X$ ml culture in the fermenter, and not in 20 ml. The dilution factor at the beginning is thus lower than the final one after uniform mixing of the honey. When a uniform mixing is attained after few minutes, the curve rebounded to a true stable level achieved by the corresponding treatment. The stable levels of the *p–t* curves after treatment, not the initial troughs, were considered in the calculation of percentage residual heat production rates. Percentage residual heat production rate is the ratio of the values after treatment and before treatment, multiplied by 100.

As the number of *p–t* curves for all bacterial species and treatments is too large to present as figures, the residual heat production rates versus honey dilution factors are presented as dose–response curves (Fig. 2a and b). In addition, MIC values obtained by the calorimetric method are compared with those from petridish bioassay and spectrophotometric techniques (Table 1). As can be seen clearly (Fig. 2a), treatment with 1:10 HB resulted in no residual heat production rate except for *P. syringae* which had a remnant value of 20%. High diversity of antibacterial activity against the different bacterial species was observed at a 1:20 HB with *B.*

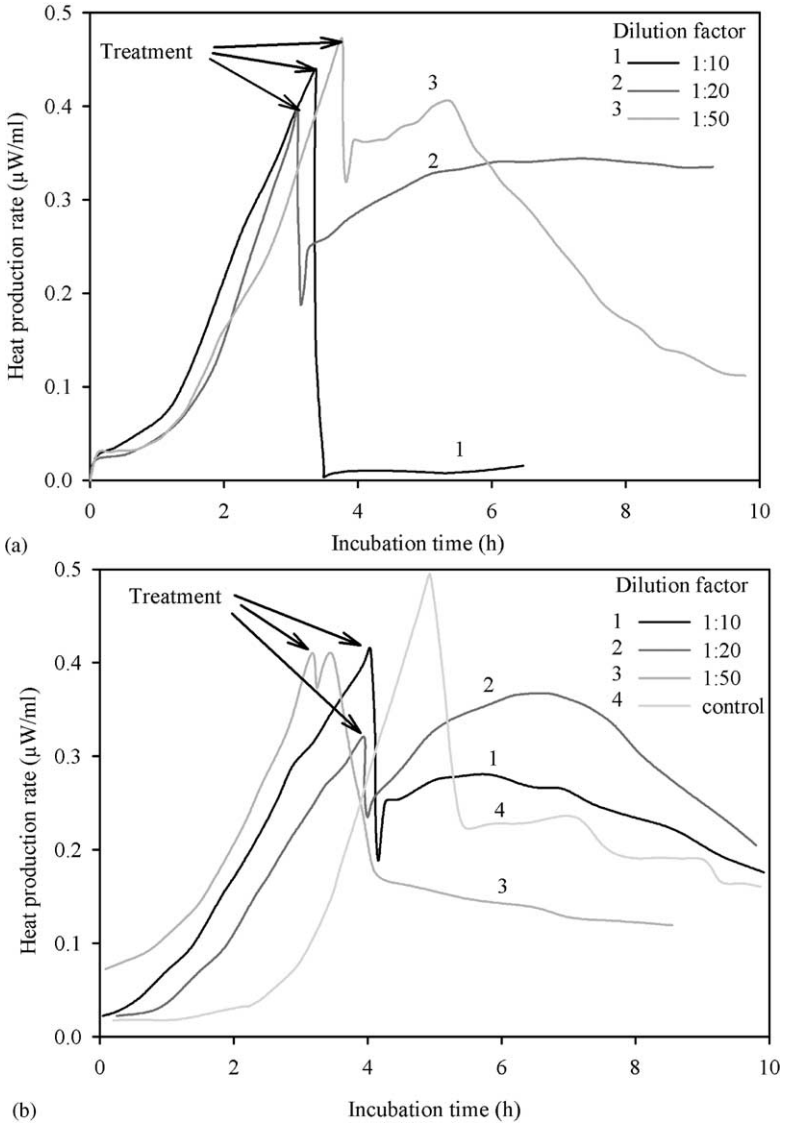

Fig. 1. Effect of treatment of the bacterium *B. megaterium* with different dilutions of: (a) Honey B; and (b) Honey T of the stingless bee *Trigona* spp. on the *p–t* curve.

megaterium displaying a residual heat production rate of up to 65%, whereas *B. subtilis* and *B. brevis* showed no life activity (Fig. 2a). The antibacterial activity of 1:100 HB was almost negligible. In case of treatments with HT (Fig. 2b), however, the sensitivity of bacteria changed from a residual heat production rate around 60%, except for *B. brevis* with 22%, after treatment with 1:10 HT to 85 to 100% for 1:100 HT.

Table 1
Minimal inhibitory concentration (MIC) of two honey samples of the stingless bee *Trigona* spp. against various bacterial species determined by petridish bioassay, spectrophotometry at 546 nm, and calorimetry

Bacteria	HB			HT		
	Petridish	Spectrophotometry	Calorimetry	Petridish	Spectrophotometry	Calorimetry
B. brevis	$1 < MIC \leq 5$	$2 < MIC \leq 5$	$1 < MIC \leq 2$	$5 < MIC \leq 10$	$5 < MIC \leq 10$	$1 < MIC \leq 2$
B. subtilis	$5 < MIC \leq 10$	$2 < MIC \leq 5$	≤ 1	$20 < MIC \leq 50$	$5 < MIC \leq 10$	$2 < MIC \leq 5$
B. megaterium	$5 < MIC \leq 10$	$2 < MIC \leq 5$	$1 < MIC \leq 2$	$20 < MIC \leq 50$	$5 < MIC \leq 10$	$2 < MIC \leq 5$
M. luteus	$20 < MIC \leq 50$	$2 < MIC \leq 5$	$1 < MIC \leq 2$	$20 < MIC \leq 50$	> 10	$2 < MIC \leq 5$
E. coli	$20 < MIC \leq 50$	>10	$1 < MIC \leq 2$	$20 < MIC \leq 50$	> 10	$2 < MIC \leq 5$
P. syringae	$20 < MIC \leq 50$	$2 < MIC \leq 5$	≤ 1	$20 < MIC \leq 50$	$5 < MIC \leq 10$	$1 < MIC \leq 2$

The values are percentage concentration of honey. Mean, $n = 3$.

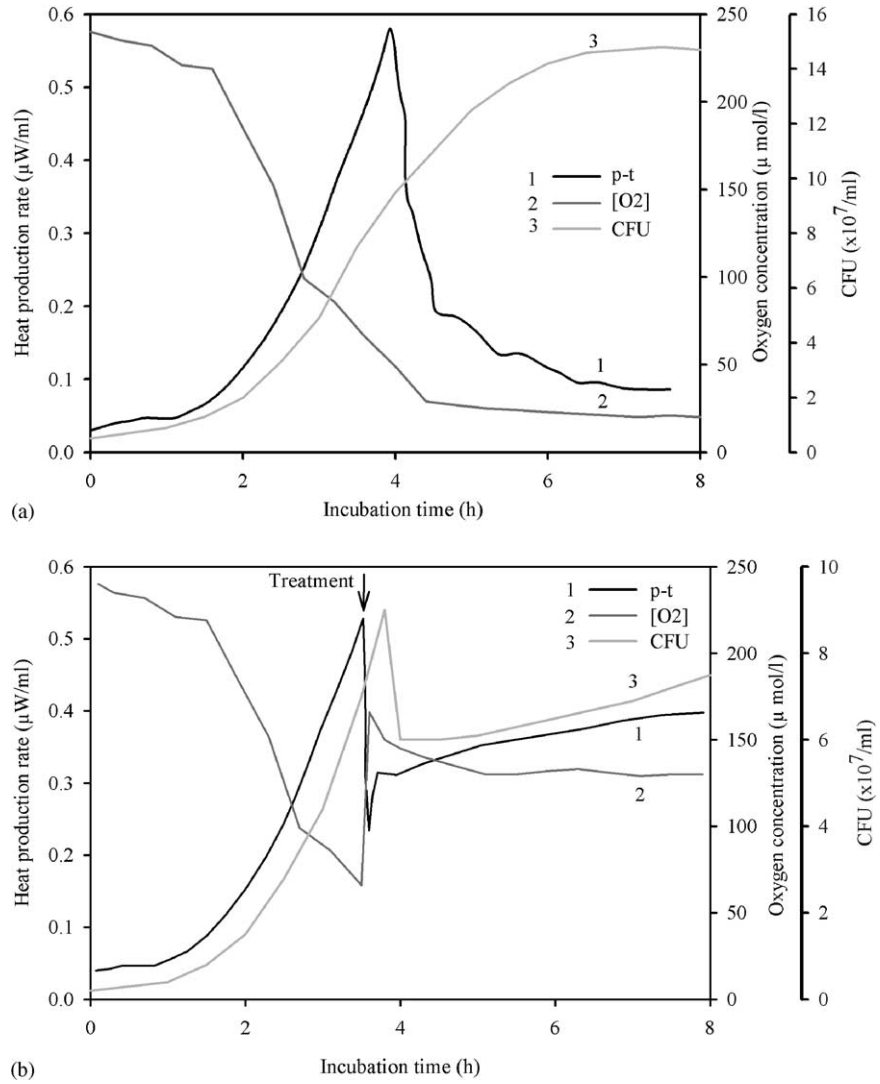

Fig. 2. Residual heat production rates of different bacterial species after treatment with various concentrations of: (a) Honey B; and (b) Honey T of the stingless bee *Trigona* spp. presented as percentage of the heat production rate after divided by that before treatment. Mean values, $n = 3$.

Simultaneous recording of the heat production and oxygen consumption rates together with counting of the number of colony forming units was done for *M. luteus*, as a representative of the obligate aerobic bacteria (Fig. 3) and for *E. coli*, a facultative anaerobe, displaying a unique *p–t* curve (Fig. 4). These simultaneous recordings were mainly done to evaluate the reliability of the calorimetric method.

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 for heat production rate and the cell number and an exponential drop for oxygen tension. These events continued similarly up to the peak of heat production rate. After the peak the heat production rate dropped steeply and oxygen concentration in the flow-line continued dropping to a constant concentration of 20 μmol l^{-1}. During this time the number of CFU increased till to the stationary phase with a cell density nearly twice as much as that at the peak of the *p–t* curve 2 h before. The *p–t* curve of *E. coli* (Fig. 4) showed a unique feature in that the heat production rate dropped from a peak of 0.62 μW ml^{-1} to 0.21 μW ml^{-1} and rose again to a plateau phase of about 0.5 μW ml^{-1}. During the whole period cell density rose continuously till to a stationary phase with 12×10^7 CFU ml^{-1}. The oxygen concentration in the flow-line dropped continuously until it achieved a constant concentration of 20 μ mol l^{-1} (Fig. 4a).

After treatment of *E. coli* and *M. luteus* with 1:20 HB, the heat production rates and numbers of CFU dropped and the concentration of oxygen increased (Figs. 3b and 4). Following the drop in the heat production rate and CFU of *M. luteus* to lower values, both stayed at a plateau level for some time and started increasing whereas the oxygen concentration in the flow-line dropped gradually (Fig. 3b). After the *p–t* curve of *E. coli* dropped drastically due to treatment at the exponential growth phase, it remained at about 0.03 μW ml^{-1} with a corresponding CFU of 1.5×10^7, whereas the con-

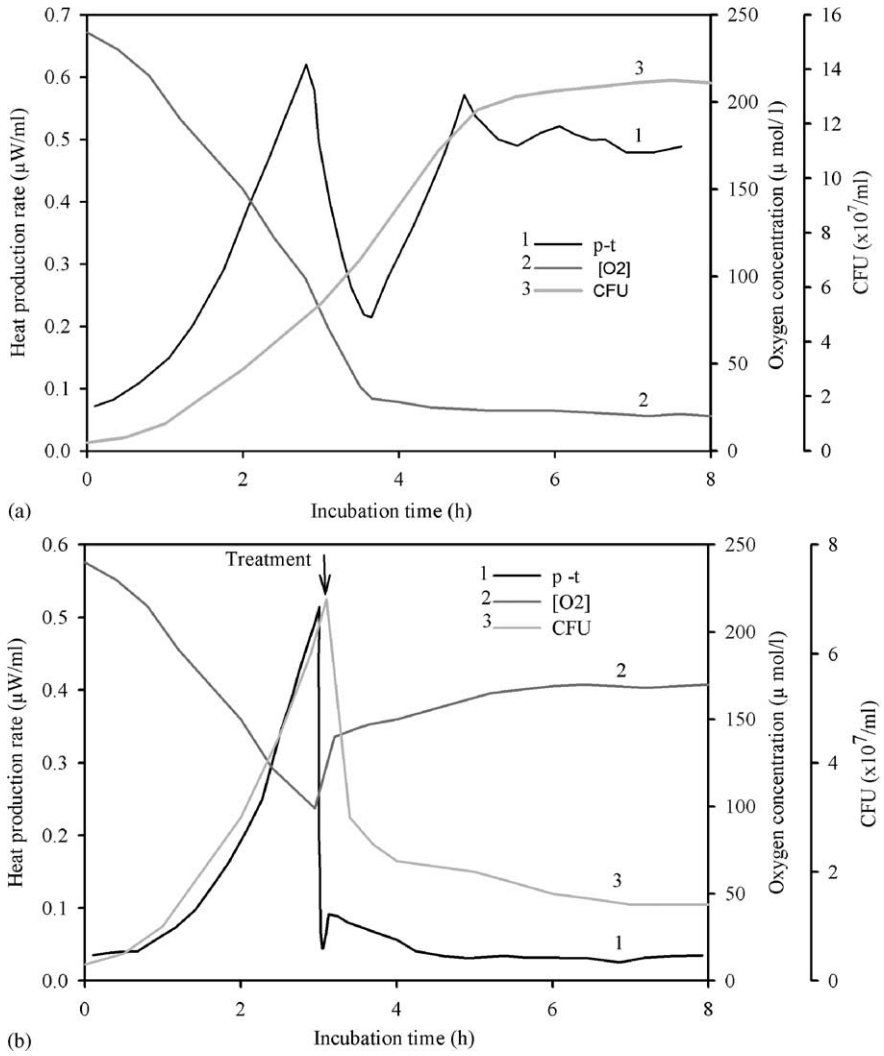

Fig. 3. Typical time course of volume specific heat production rate (p), oxygen concentration [O_2], and number of colony forming units of the bacterium *M. luteus*: (a) without treatment (control); and (b) after treatment with a 1:20 diluted Honey B of the stingless bee *Trigona* spp.

centration of oxygen remained at a higher level at about 170 μmol l^{-1}. In general, the treatment with 1:20 HB caused an immediate increase in the concentration of oxygen in the flow-line, and corresponding decreases in the number of CFU and the heat production rate of both bacterial species.

4. Discussion

The calorimetric results clearly demonstrated the mode of action of the two honey samples. High concentration of HB (1:10 dilution) displayed bactericidal activity dropping the *p–t* curve to the baseline immediately after treatment keeping it at this level for the rest of the experimental period (Fig. 1a). As the dilution factor doubled to 1:20 the nature of antibacterial activity changed from bactericidal to bacteriostatic. The bacteriostatic activity is displayed by a plateau phase of the *p–t* curve after treatment, achieved after an initial drop of the heat production rate. The treatment of an actively metabolizing and growing culture reduces the heat production rate to a lower value since the physiological status of the culture changes from a reproductive plus basic metabolism to basic metabolism alone. As the bacterial culture treated with the bacteriostatic concentration of honey, 1:20 HB, shows only a metabolism of maintenance, the heat production rate remains at a constant level. The antibacterial activity of HT is mainly bacteriostatic, inhibiting cell growth (Fig. 1b). The gradually decreasing heat production rate of the bacterial culture after treatment with different concentrations of HT could be an indication that cells are dying with a prolongation of the incubation time. This mode of bacteriostatic action is typical for some antimicrobial agents that act on the cell wall and cell membrane of the bacteria by disrupting the integrity of these structures making the cell "leaky" and inhibiting their repair by hindering synthesis of new cell membrane/cell wall components, leading to gradual death. The disruption of cell membrane and cell wall integrity by some antimicrobial agents, such as antibiotics

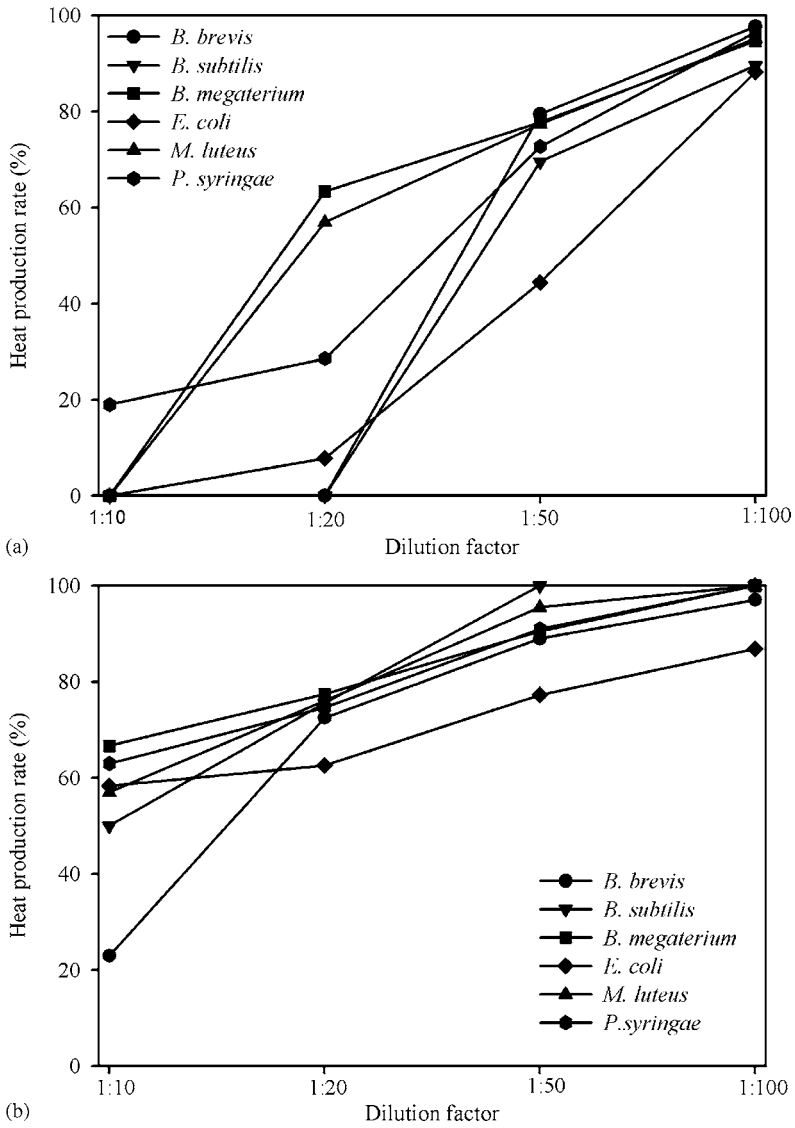

Fig. 4. Typical time course of volume specific heat production rate (p), oxygen concentration [O_2], and number of colony forming units of the bacterium *E. coli*: (a) without treatment (control); and (b) after treatment with a 1:20 diluted Honey B of the stingless bee *Trigona* spp.

that mainly act against Gram-positive bacteria by inhibiting peptidoglycan synthesis and hence disrupting cell wall integrity, is an already established fact. This phenomenon of disrupting cell wall integrity by the honeybee product, propolis was demonstrated by us against the Gram-positive bacterium *M. luteus* [9].

The dose–response curves obtained in the calorimetric experiments demonstrated that the antimicrobial activities increase with increasing concentration of both honey samples. Comparison of results from the two honey samples shows that HB has a stronger antimicrobial activity than HT. It might be due to the fact that it acts both as bactericidal at higher and bacteriostatic at lower concentrations, where as HT is mainly bacteriostatic against the bacterial species tested.

Since the calorimetric vessel and the fermenter are separated by an unavoidable geometric distance, a difference in the physiological status of the organisms in the fermenter and in the flow-line could be provoked under stress conditions. The simultaneous recording of heat production rate, oxygen concentration in the flow-line and CFU demonstrates that the p–t curve is a true picture of the metabolism in the fermenter only in the first hours before the p–t curve attains its peak. During this period heat production rate and number of CFU increase while oxygen concentration in the flow-line decreases significantly (Figs. 3a and 4a). Later on, the calorimetric curve is no more a good indicator of the metabolic activity in the fermenter due to depletion of oxygen in the flow-line [9,10]. Thus, the calorimetric death phase is actually the late exponential and stationary growth phase of the bacterial culture in the fermenter, as displayed by the simultaneously monitored cell density.

The MIC values obtained for a certain honey sample against a given bacterial species were highly affected by the

method employed. The petridish bioassay method is the less sensitive of the three methods used followed by the spectrophotometric method. The calorimetric method showed the highest sensitivity displaying even the antibacterial activity of very low honey concentrations (Table 1). One of the superior qualities of the calorimetric method is not only to elucidate the antimicrobial activity of very low honey concentrations but also to display the mechanism of antibacterial action online. This is unlike the cumulative effects of long time incubation such as 30–60 min for spectrophotometry and 24–48 h for petridish bioassay. In addition to that, several other problems encountered by the other two methods do not affect the calorimetric results. Results from the petridish assay method are affected by agar layer consistency, incubation temperature, polarity, and diffusion potential of the test substance or inoculum size. Spectrophotometric results are affected by inoculum size and color of the test substance. At first glance, it seems that the inoculum size affects the calorimetric results too, but it does not have any influence. Treatment is usually done at the logarithmic growth phase and cell density can exactly be determined at any point on the *p*–*t* curve in this phase. Thus, treatment can be done at a precisely determined cell density of the culture. The only role played by the size of the inoculum is on the length of the lag phase, which has nothing to do with the treatment at the logarithmic growth phase.

The only limitations observed with the calorimetric method are that (i) only one sample can be investigated at a time and hence it is time-consuming; (ii) it needs expertise and hence the chance of introducing error is high unless care is taken; (iii) it is expensive to use in routine laboratory investigations; and (iv) it should be used in connection with other methods such as polarography if investigation is to be done at higher cell densities. Therefore, for non-routine investigations mainly to tackle problems that could not be solved with the other methods, it is a very reliable and robust technique.

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