CALORIMETRIC INVESTIGATION OF THE ACTION OF PROPOLIS ON BACTERIAL GROWTH AND METABOLISM

A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGY

BY

ASSEGID GAREDEW

JUNE, 1996

ACKNOWLEDGEMENTS

I am highly indebted to my research advisor Prof. Dr. I. Lamprecht for allowing me to do the experimental part of this study in his laboratory at the Free University of Berlin, Institute for Biophysics, Germany. It is with great pleasure that I express my heartfelt gratitude and sincere thanks for his consistent guidance, keen interest and warm welcome to discuss issues of relevance to this study.

I am also very grateful to all members of the research group of Prof. Dr. I. Lamprecht, especially Prof. Dr. B. Schaarschmidt and Dr. R. Hölzel for their warm welcome, hospitality and readiness to open discussion with me. I would also like to thank Prof. Dr. Gunda Kraepelin, Technical University of Berlin, Institute for Microbiology, for kindly providing me with the organism for this work and the Pharmaceutical Institute of Free University of Berlin for providing me with the propolis samples 1 and 2 (prop1 and prop2).

I would like to express my sincere thanks to my advisor Dr. Fassil Assefa for his valuable suggestions and discussions during the write-up of this thesis.

I am very much indebted to my colleagues Ato Amare Gessesse, Dr. Dawit Abate and Dr. Wendimagegn Mammo for their valuable comments.

I would like to thank my friends, especially Ahmed Mustefa, for all the encouragement and support particularly during the write-up of this thesis.

I also thank the Holleta Bee Research Centre for providing me with propolis sample 3 (prop3).

The financial support from DEUTSCHER AKADEMISCHER AUSTAUSCHDIENST (DAAD) that enabled me to pursue my studies both in Addis Ababa and Germany is gratefully acknowledged.

TABLE OF CONTENTS

CONTENTS PAGE

ACKNOWLEDGMENTS i
TABLE OF CONTENTS ii
LIST OF FIGURES iv
LIST OF TABLES vi
ABSTRACT vii

1. INTRODUCTION 1

2. LITERATURE REVIEW 4
   2.1 CALORIMETRY 4
      2.1.1 General review 4
      2.1.2 Heat conduction calorimeters 7
         2.1.2.1 Principles of measurement and dynamic correction 8
      2.1.3. Applications of calorimetry 15
         2.1.3.1 Analytical tool 15
         2.1.3.2 Quantitative tool 17
   2.2 PROPOLIS 18
   2.3 OBJECTIVES OF THIS WORK 22

3. MATERIALS AND METHODS 23
   3.1 BIOLOGICAL MATERIAL AND GROWTH CONDITIONS 23
   3.2 EXTRACTION OF PROPOLIS 24
   3.3 CALORIMETRY 25
      3.3.1 Sterilization of the flow line 26
      3.3.2 Calibration of the calorimeter 26
      3.3.3 Calorimetric cultivation of cells and effect of EEP 27

3.3.4 Treatment of the P-t curve 28
3.4 POLAROGRAPHIC OXYGEN SENSORS (POS) 29
3.5 AGAR WELL DIFFUSION ASSAY 31

4. RESULTS 33
4.1 DETERMINATION OF OPTIMUM GROWTH TEMPERATURE 33
4.2 CELL NUMBER VERSUS OPTICAL DENSITY 34
4.3 PROPOLIS EXTRACTION 34
4.4 CALORIMETRY 36
4.4.1 Electrical calibration 36
4.4.2 Effect of inoculum density on the P-t curve 37
4.4.3 Relationship between the P-t curve and other features of the culture 39
4.4.4 Effect of EEP on the P-t curve 42
4.4.4.1 Effect at different phases of the P-t curve 42
4.4.4.2 Comparison of effect of different EEP at the mid exponential phase of the P-t curve 46
4.4.5 Comparison of effect of water soluble and insoluble components of EEP 51
4.4.6 Effect of ampicillin 54
4.4.7 Microscopic examination of cell morphology 56
4.5 AGAR WELL DIFFUSION ASSAY 56

5. DISCUSSION 61

6. CONCLUDING REMARKS 70

7. REFERENCES 72

LIST OF FIGURES

FIGURE PAGE

Fig. 1. Schematic diagram of a twin heat conduction calorimeter used in the present work. 10

Fig. 2. Power-time curve obtained from a heat conduction calorimeter measuring a constant heat production rate (P) released in the interval t_1 to t_2. 14

Fig. 3. Effect of temperature (°C) on the generation time (h) of *M.luteus* 33

Fig. 4. Plot of OD (at 646 nm) versus cell number mL^{-1} for *M. luteus* grown at 30 °C. . 34

Fig. 5. Weight ($g\ mL^{-1}$) of the different components of EEP obtained by taking 1 mL of 10% EEP and mixing with 9 mL distilled water to precipitate out the water insoluble components. 35

Fig. 6. Plot of the calorimetric voltage signal generated as a function of the power supplied for the electrical calibration of the flow microcalorimeter to determine its sensitivity (S) 37

Fig. 7. Heat production rate, P, as a function of incubation time for the flow microcalorimetric growth of *M. luteus* 38

Fig. 8. Flow microcalorimetric growth of *M. luteus* and subsequent changes in features of the culture 40-41

Fig. 9. Effect of propolis tincture at different phases of the P-t curve of *M. luteus* in a flow microcalorimeter. 43-45

Fig. 10. Effect of EEP of prop1 (A); prop2 (B); prop3 (C); and tincture (D) on the P-t curve of flow microcalorimetric growth of *M. luteus*. 47-48

Fig. 11. Dose-response curves for EEP of prop1 (a); prop2 (b); prop3 (c); and tincture (d) added at the mid exponential phase of growth of *M. luteus* in a flow microcalorimeter 50

Fig. 12. Effect of water soluble component, water insoluble component, and unprecipitated EEP of prop1 (A); prop2 (B); prop3 (C) and tincture (D) on the P-t curve of *M. luteus* grown in a flow microcalorimeter 51-53

Fig. 13. Effect of ampicillin sodium salt on the P-t curve of *M. luteus* grown in a flow microcalorimeter. 55

Fig. 14. Dose-response curves for the antibacterial activity of prop1 (A); prop2 (B); prop3 (C); tincture (D); and ampicillin (E) for *M. luteus* using the agar well diffusion assay method. 57-59

LIST OF TABLES

TABLE PAGE

Table 1. Result of electrical calibration of the flow calorimeter by a heater of resistance, R = 49.479 Ohm and at a pumping rate of 100 mL h^{-1}. 36

Table 2. Effect of inoculum density on Q_t (J mL^{-1}) during the growth period and cell density at the main peak (cells mL^{-1}) of the P-t curve of *M. luteus* growing in a flow microcalorimetric system. 39

Table 3. Drop in power output (mW mL^{-1}) achieved due to incorporation of 1 mg mL^{-1} different propolis preparations at the mid exponential phase of growth of *M. luteus* in a flow microcalorimeter. 54

ABSTRACT

Power-time (P-t) curves were established by growing *Micrococcus luteus* in a flow calorimetric system using different inoculum densities to see if inoculum size affects calorimetric experiments. The P-t curves obtained for the lower inoculum densities used were the same in several aspects except length of the calorimetric lag phase. Spectrophotometric and polarographic experiments were done simultaneously with the calorimetric experiments to see if results from the latter are true pictures of metabolic events in the fermenter or experimental artifacts introduced due to separation of fermenter from the calorimeter. Results from experiments with the different methods showed the same pattern indicating that separation of fermenter from the calorimeter does not introduce error at the pumping rate used (100 mL h^{-1}).

Extraction of propolis was done with 70% methanol as a solvent. The yield of extraction was 21% (w/w) for all propolis samples used. Addition of water to the ethanol solution of extract of propolis (EEP) resulted in precipitation of the water insoluble components and hence separation of the water soluble from the water insoluble components. The proportion of water soluble components was very low in propolis sample 3 (prop3).

Calorimetric experiments with propolis were done by incorporating increasing concentrations of tincture at different phases of the P-t curve and different propolis samples at the mid exponential phase of the P-t curve for comparison of their effect. It was found that effect of propolis can be detected at all phases of growth. However, comparison of antibacterial activities for the different concentrations of propolis used was not possible at the lag phase.

Variation in activity was observed among the different propolis samples in their effect on the drop of the P-t curve which is directly proportional to the amount of cells killed or inactivated, and the time needed for the resumption of growth after the curve has fallen off to a minimum value. Though incorporation of tincture resulted in a drop of the P-t curve comparable to other propolis samples, growth was resumed sooner suggesting lower inhibitory effect on the survivors. But propolis sample 2 (prop2) possesses higher killing and inhibitory effect on the survivors.

For all propolis samples used the strength of antibacterial activity, based on drop of the P-t curve and subsequent effect, could be ordered as follows: EEP > water insoluble component > water soluble component. For some propolis samples the effect of EEP was greater than even the sum of two of its components at a certain concentration. It was therefore deduced that the water soluble and insoluble components exhibit synergistic interactions in EEP.

The calorimetric method was found out to be slightly less sensitive to detect effect of lower concentrations of propolis than the agar well diffusion assay method. However, the former method had a very short assay time and clearly demonstrated that the mechanism of action of propolis is bactericidic.

1. INTRODUCTION

Mankind has always been affected by disease which at times, in the form of pestilence or plague, has spread throughout the entire community. Until the end of the last century, only two types of diseases were cured by chemotherapy (Baldry, 1967). These were: (i) healing of syphilis by mercury which was started by Paracelsus around the beginning of the 16th century and was in use until it was substituted by certain arsenical compounds, discovered at the beginning of this century; and (ii) curing of malaria by quinine which was started at the beginning of the 19th century. Quinine was isolated from the bark of cinchona trees by Pelletier and Coventou. Control of the spread of most of the other diseases, at that time, was tried by isolating a patient from the population, by folk medicine, and sometimes vaccination, a technique introduced at the end of the 18th century by Jenner for the prevention of small pox.

In recent years, however, all this is changed, at least in developed countries, with the discovery and/or synthesis of drugs effective against a range of pathogenic microorganisms. The identification of bacteria under the microscope towards the end of the last century stimulated Ehrlich to search systematically for chemical substances which when taken into the body, would kill harmful bacteria, while doing no damage or as little damage as possible to the host tissue. This idea, which is in use today too, led to the discovery of different antimicrobial drugs from microorganisms, plants, animals, insects, minerals etc., or even by artificially synthesizing them. The discovery of new drugs will broaden the variety of bacterial and fungal infections susceptible to treatment by chemotherapy. In addition, attention has particularly been payed to the problem of overcoming microbial resistance to drugs by the introduction of new substances or the development of combined therapy using two or more antimicrobial drugs simultaneously (Buxton and Fraser, 1977). The search also continues on an increasing scale for agents in the control of protozoal infections, viral diseases, and in the treatment of tumours. The discovery of drugs with one or more of these functions is of advantage (Wright and Montag, 1959). There are some types of drugs, synthetic or natural, effective against different types of pathogenic microorganisms and other types of diseases. Propolis is one of such drugs obtained from the natural environment and possessing versatile biological activities including antibacterial, antiviral, fungicidal, immunostimulating, hypotensive, cytostatic (Ghisalberti, 1979), antiinflammatory (Wang *et al.*, 1993) and antiulcer activities (Scheller *et al.*, 1989).

Propolis is a natural product collected from different tree parts and slightly modified in the bee hive by honey bees mainly by the addition of enzymes and wax (Greenaway *et al.*, 1987). It is found in most places where bees are found and hence available even in places where availability of other drugs is limited. Since it can be collected from the natural environment it has a very low cost of production. The cost of production of a drug is an important factor that limits its extensive use in a community due to economic reasons. Hence, cheaper but highly effective drugs, mainly from the natural environment such as propolis are desirable to use.

The screening for new antimicrobial drugs from different sources occupies the attention of a large number of workers concerned. The approaches to this problem, as to the source of a drug, can be as many as there are laboratories concerned. Many such survey programmes start with an *in vitro* test in an effort to determine the activity of a compound. *In vitro* tests can be done using the following methods.

i. **Agar well diffusion/ agar plate**, dilutions of the antimicrobial drug to be tested are placed in a well or on the surface of an agar plate, inoculated with the test organism, in a form of cubes or impregnated on a filter paper. The drug will diffuse in to the medium during subsequent incubation, and will cause zone of inhibition in the growth of susceptible organisms. A number of factors such as strength of the agar, solubility and rate of diffusion of the drug, length of time and temperature value under which the test is carried out affect results from this method. Regardless of these limitations, this method is still in use, sometimes the sole classical microbiological technique, to test the effect of coloured drugs or drugs such as propolis that cause turbidity when mixed with the growth media such as propolis.

ii. **Serial dilution**, involves adding a standard volume of broth suitable to support growth of the organism to be tested in to a series of culture vessels. To the series is added increasing concentration of a drug. A known cell density of an actively growing culture is added to each vessel and incubated for a certain time. The culture is then examined for the inhibition of growth spectrophotometrically. This method is applicable only for those drugs that do not interfere with absorbance when mixed with the growth media in use.

iii. **Polarography**, manipulates rate of oxygen uptake and hence aerobic metabolism of a culture. It is among the relatively recent biophysical methods used to investigate action of metabolic inhibitors/ stimulators on growth and metabolism of a culture. In this method granular particles must be absent, which otherwise could precipitate on the membrane surface

thus blocking the oxygen diffusion path. Therefore, even if it is a relatively recent method manipulating aerobic respiration of a culture it has a serious limitation with solutions/ media that posses components which can easily precipitate out.

iv. **Calorimetry**, this is a relatively recent method for the investigation of effect of antimicrobial drugs on metabolism and growth. It is not limited by the problems encountered in the other three methods mentioned above. Since this method manipulates net heat release by a culture, it offers appropriate information as to the metabolic behaviour of a culture with in a short period of time. Continuous recording of the heat released by a culture from the point of addition of a drug till the end of the experimental period tells whether action of a drug is bacteriostatic or bactericidic and also exhibits about the kinetics of action of a drug. These phenomena can also be revealed by the polarographic method and to a lesser extent by the serial dilution method if a continuous recording is done.

Though both calorimetry and polarography manipulate metabolism the former method has advantages over the latter. Since calorimetry measures net heat release it exhibits both aerobic and anaerobic respiration. However, polarography deals only with aerobic respiration and its application is restricted to aerobes. In addition, calorimetry is not limited by nature of the media used. The introduction of such methods, with less limitations, in the assay of antimicrobial drugs may increase the chance of discovery of new drugs and may also help in the study of mechanism of action of drugs.

In the present work calorimetry was used to investigate the antibacterial activity of propolis. Investigation of antibacterial activity of propolis by the serial dilution and polarographic methods was not possible due to the formation of turbid suspension when propolis was mixed with water. The agar well diffusion assay method was used in comparison with the calorimetric experiments.

The introduction of natural preparations such as propolis, with less side effects, in medicine may increase the range of bacterial, fungal, viral and other diseases under control.

2. LITERATURE REVIEW

2.1 CALORIMETRY

2.1.1 General review

All physical, chemical, or biological processes in nature are accompanied by heat evolution or absorption, which can be measured by Microcalorimetry (Spink and Wadsö, 1976).

Energy, in order to be measured, must produce some effect which can be sensed and measured. The effect invariably produced by thermal energy is the heating of a colder mass by a hotter one. The effect produced by non-thermal energy is to cause one mass to act upon another in such a way that something happens of a chemical, electrical, kinetic, mechanical, or radiant nature. Energy can therefore be considered as something "in transition" that is capable of being exchanged, as a result of which some effect is caused (Hemminger and Höhne, 1984). Thus, heat is thermal energy that is exchanged between a hotter and a colder mass because of a difference in temperature between them. Therefore, the form of energy known as heat can only be conceived as coupled with an exchange of energy (Spink and Wadsö, 1976). Heat is always associated with heat flux. The concept of flux or exchange between two systems emphasizes the quantitative nature of energy in general and of heat, as a form of energy, in particular. Heat is thus the amount of energy exchanged with in a given time interval in the form of heat flux. Calorimeters are the instruments used for measuring this heat.

Following the establishment, on a chemical basis, of the molecular nature of matter two different, though related, approaches were made toward correlating the phenomenon of heat with ordinary mechanical energy. One of these works was the kinetic approach developed through the work of Clausius, Boltzman and Maxwell, and the other was the statistical mechanical approach of Gibbs (Battley, 1987). In the theories advanced by both of these schools, thermal energy is considered to be a random molecular motion which is different from ordinary mechanical motion only in that the measuring instruments used in thermodynamic investigations are not sufficiently sensitive to enable random molecular motions to be perceived. Indirect evidence for this motion exists in the form of Brownian motion of particles of colloidal size. Similarly, the random motion of molecules comprising an otherwise

motionless mass can be ordinarily only indirectly perceived through the sensing of temperature. Heat is thus considered as the kinetic and potential energy of random molecular motion and the conversion of mechanical energy into heat is simply the randomization of the energy of bulk masses of molecules that had been previously associated with an average motion in a particular direction, or an average location in a particular spatial position (Hemminger and Höhne. 1984).

The production of heat by living organisms is one of the transformations of energy brought about by their cells. Calorimetry is a measure of the algebraic sum of the enthalpy changes of all biological processes in the cell (James, 1987). The energy consuming reactions of anabolism are negligibly small compared with those of energy liberating reactions of catabolism in a living cell, so that in all cases of cellular reactions a net production of heat results. In every day life this is manifested by the rise in temperature of fermenting liquids, decomposing manure and other organic materials such as straw. The heat evolved during the bacterial degradation of organic substances can have disastrous consequences, for example in the spontaneous ignition and combustion of refuse or hay heaps as a result of overheating. During the production of organic solvents, antibiotics, etc. by fermentation, a tremendous amount of heat is generated; this has to be removed. Thus any information relating to the amount of heat energy produced and its rate of production is important (Lamprecht, *et al.* 1991).

Since many metabolizing systems studied in biology exhibit a gas consumption or production, calorimetry can be compared with manometric and polarographic methods (Lamprecht, 1983). It was some two hundred years ago that Crawford clearly stated, for the first time, that oxygen consumption is roughly proportional to heat production: the fundamentals of modern indirect calorimetry (Blaxter, 1978). The sentences of Crawford, namely that there is a good correlation between oxygen consumption and heat production, led to the opinion that energy metabolism is a biological combustion of energy rich compounds at low temperature and that direct calorimetry may be substituted by the cheaper and easier indirect ones, such as O_2 consumption and CO_2 production (Lamprecht, 1985b).

The history of calorimetry dates back to the 18th century, i.e. about 100 years before Clausius formulated the first and second laws of thermodynamics. The first quantitative measurements were probably those made by Black in 1760 who measured heats of melting of ice and realized that the heat delivered to melting ice serves for transition from solid to liquid

state at a constant temperature (Hemminger and Höhne, 1984). Thus the distinction between the concepts of temperature and heat, which is accepted today, was made for the first time. Although biology stood at the cradle of calorimetry two hundred years ago, only recently and particularly since the development of sensitive calorimeters during recent decades that calorimetry has found widespread use in such areas as medicine, biology and biophysics (Lamprecht, 1985a). Systems under investigation have ranged from simple model compounds to whole animals, plants and even ecosystems (Beezer, 1980; James, 1987; Jones, 1979). The first recorded calorimetric experiment on microorganisms was the heat of fermentation of yeast developed in a large brewer's vat of 21,000 litre by Dubrunfaut around the mid of the 19th century (James, 1987). This can't, however, be considered as microcalorimetry since the term involves calorimeters that detect very low power out put in the order of μW. The first true microcalorimetric measurements during growth and fermentation of yeasts were made by Rubner at the beginning of this century (Hemminger and Höhne, 1984).

One of the first functioning reaction calorimeters was constructed by Lavoisier and Laplace at the end of the 18th century. In this calorimeter, samples were placed in a central cage surrounded by ice, and hence the name **ice calorimeter**. The amount of heat evolved could then be calculated from the amount of melted ice since the former is proportional to the mass of the latter, Δm:

$$\Delta Q = C_t . \Delta m$$

where ΔQ is the heat exchanged, C_t is the specific heat of transition, and Δm the mass of melted ice.

A disadvantage of all **phase transition calorimeters** including the ice calorimeter stems from the fact that the experimental temperature is determined by the phase transition temperature of the substance used (Gravelle, 1977). Consequently, a variety of experimental temperatures can only be obtained in such calorimeters by using substances other than water. The advantages of phase transition calorimeters lie in their relatively simple construction, their great sensitivity and the possibility of enclosing the calorimeter in a vessel in which a phase transition identical to that occurring in the calorimeter takes place. This latter approach compensates for disturbances from the surrounding.

In the more or less **adiabatic** calorimeters of Crawford, Regnault, Hess and Berthelot, the reaction heat is retained and calculated from the resulting temperature change in the calorimetric vessel. The calibration value of such calorimeters is therefore dependent on the

heat capacity of the calorimeter plus its contents. These calorimeters (nowadays often termed as **isoperiobolic** reaction calorimeters since they are surrounded by isothermal jackets) are still frequently used, in particular the type designed by Sunner and Wadsö (1959).

In the thermopile **heat conduction calorimeter** constructed by Tian (1923) and further developed by Calvet (1948) the heat generated or consumed in the calorimetric vessel initially causes a change of temperature with regard to the surrounding. This temperature difference over the thermopile wall generates an electromotive force (EMF) which is proportional to the heat flux. The EMF generated is presented on a recorder, via an amplifier, as the heat absorbed or released per unit time. The EMF over the thermopile wall causes a relaxation process: a heat exchange with the surrounding (external heat sink) of a relatively high heat capacity, via a battery of thermocouples (i.e. thermopiles), continues until the re-establishment of isothermal or steady state conditions.

Since instruments of the heat conduction type are essentially **isothermal**, they are suitable for use in studies on living systems, for example, where it is necessary to follow heat production over extended periods (Wadsö, 1987). This is due to the fact that living organisms have a certain temperature optimum for their normal functioning which can be maintained through out the experimental time by the isothermal calorimeters. Although the Tian-Calvet calorimeters are very sensitive 'Watt-meters' whose sensitivity is of the order of μW, they have a relatively slow kinetic response (the time constant, τ, being approximately 10 min). This is due to the fact that the thermocouples used have a relatively high thermal resistance. Nowadays, however, the time constant is as small as 96 seconds for the calorimeter used in the present work.

Later developments in the field of calorimetry were made by Wasdö and co-workers who constructed a series of heat conduction calorimeters, among them the microcalorimetric system produced by LKB (Bromma, Sweden) used in the present work. Calorimeters of different types have been treated broadly in reviews by e.g. Armstrong (1964); Sturtevant (1971); and more recently by Wadsö (1990).

2.1.2 Heat conduction calorimeters

In the present work, all calorimetric measurements have been done with an essentially

isothermal twin heat conduction calorimeter which will be described in some detail together with some measuring principles for heat conduction calorimeters in general.

2.1.2.1 Principles of measurement and dynamic correction

The heat transport phenomena: conduction, radiation, convection and heat transfer are of central importance in calorimetry. On the one hand, the occurrence of temperature difference causes a heat flux and thus creates a possibility of heat leaks - namely, heat fluxes not detected by the measuring sensor; on the other hand, no heat exchange can take place in the absence of a temperature difference. The experimenter finds himself facing a dilemma: in order to be measured, heat must be made to flow, but a heat flux is associated with temperature difference which creates errors in measurement, i.e. heat leaks. There are two possible ways out of this dilemma: the adiabatic calorimeter and the twin device.

In the adiabatic calorimeter, as described previously, suitable measures are taken to prevent any escape of heat from the measuring system to the environment. For this purpose the measuring system is surrounded by a shield which has exactly the same temperature or a shield of 'infinitely large' thermal resistance. Any heat exchange in the system brings about a change in its temperature. The shield must be so adjusted as to match exactly this temperature change or prevent any heat loss to ensure that the unknown heat exchange with the environment is reduced to a tolerable magnitude. The practical construction of this instrument is expensive and its operation very laborious; however, the quality of the obtained results is not even approximated by any other procedure (Hemminger and Höhne, 1984). However, this instrument is not as such useful to follow up heat production of living organisms over extended periods due to the subsequent rise of temperature beyond the optimum range.

The second possibility of reducing the effects of the inevitable heat leakage is the so called 'twin' device. In this technique two measuring systems that are made as equal as possible to one another are operated in common surroundings in a symmetrical arrangement. Now if the inevitable measuring errors inherent in the two systems are made to be of equal magnitude, they can be offset by differential measurements. If the heat leaks between the measuring systems and the surroundings are equal in the two systems, other disturbances such as locally homogeneous temperature fluctuations in the surroundings affect the two measuring systems to the same extent, at least in a first approximation, so that the effects of these

disturbances are mutually offset owing to the differential signal. One of these measuring systems is filled with the sample to be investigated and the other, reference vessel, is filled with a non reacting solution such as sterile water or left empty so that no heat generation or absorption can take place in the latter vessel by any sort of reaction. Before starting the experiment, i.e. when there is no heat production or consumption, the differential amplifier is set at a voltage value of zero.

Heat conduction calorimeters, usually constructed on the 'twin' principle, may be roughly divided into two classes: the batch and flow systems. In the first group, a closed vessel contains all the necessary ingredients for a reaction, and perhaps auxiliary equipments for initiating the reaction, mixing, stirring, illuminating, and so on. In flow calorimeters the reaction occurs in a separate fermenter or vessel and only a part of this solution is pumped through a tube system into the calorimeter and back to the fermenter.

Both the flow-through and batch systems offer advantages and drawbacks and should be chosen carefully for investigations. While batch instruments are inevitable in experiments on whole animals or liquid systems with coarse particles, flow calorimeters have superior efficiency in investigations of microbial cultures (Mønk and Wadsö, 1968), immobilized enzymes, and tissue cultures on glass beads or other carrier materials (Mattiasson *et al.*, 1977).

The advantage of batch experiments with microorganisms lies in the fact that the culture is enclosed within the calorimeter and that all the heat produced by metabolism during maintenance and growth is detected by the instrument. The important drawback of this method is connected with the thermal disturbance as a result of any mechanical interference with the system. Stirring to avoid sedimentation of cells, lack of homogeneity or decrease of oxygen pressure around the cells introduces heat of friction which is not completely compensated for by a similar stirring device in the twin chamber of the system. The addition of materials or removal of samples during the experiment in order to determine substrate or product concentration or cell counts disturb the thermal signal. Aeration introduces considerable heats of evaporation and the simultaneous monitoring of pH value, optical density or oxygen pressure in the medium is difficult due to the limited space in the calorimetric vessel. The use of a flow-calorimeter connected to a fermenter enables to overcome these problems (Eriksson and Wadsö, 1971), and allows investigations under true aerobic conditions (Brettel *et al.*, 1972). None of the above-mentioned activities on the liquid culture in the fermenter have any influence on the calorimetric signal. However, the crucial disadvantage of this approach lies

in the separation of fermenter and calorimeter and the necessary tube connection between the two. A minimum tube length is unavoidable due to geometrical reasons and the need for a highly effective thermal equilibration of the in-flowing liquid to the working temperature of the calorimetric cell. This leads to a considerable change in the metabolic state of a liquid volume leaving the fermenter during the transport to the calorimeter, especially at high cell densities. A drastic increase in the pumping rate and, thus, a corresponding decrease in flow times is not possible because of the necessary thermal equilibration. Thus, in some situations the calorimetric signal may no longer be a true picture of the metabolic events in the fermenter and has to be evaluated with precaution (Lamprecht, 1983 ; Monk and Wadsö, 1968).

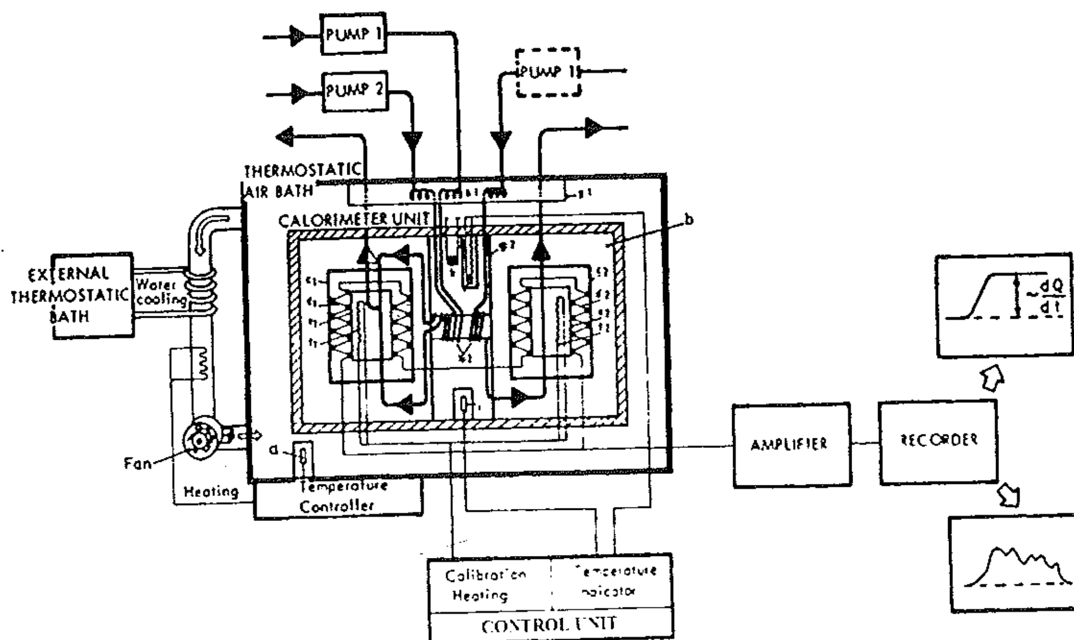


Fig. 1. Schematic diagram of a twin heat conduction calorimeter used in the present work. (a) Temperature sensor and controller of the thermostatic air bath (surrounding); (b) heat sink; (c1 & c2) detectors; (d1 & d2) thermopiles; (e1 & e2) mixing cell and reaction cell of the flow-through type, respectively; (f1 & f2) calibration heaters; (g1 & g2) heat exchangers I and II, respectively; (h1 & h2) heat exchanger coils; (i) sensor of the heat exchanger; (j) heat sink heater and (k) heat sink cooler. Redrawn from LKB-10700-1 flow microcalorimeter operation manual (LKB/ Bromma, Sweden).

In heat conduction calorimeters, as the heat produced in the calorimetric vessel passes out through the thermocouples and detected, the liquid leaves the calorimetric vessel (Poore and Beezer, 1983). Thus, the effective sensitivity of the instrument significantly changes with

the flow rate (Monk and Wadsö, 1968; Poore and Beezer, 1983). This is due to the fact that at higher pumping rates some amount of the heat produced in the calorimeter may be pumped out with the out-going liquid without being detected. However, at lower rates most of the heat passes through the thermopile and is hence detected. In fact, this is not as such a serious problem since electrical calibration is done at the experimental pumping rate and the calibration factor is involved in the evaluation of experimental results.

Heat evolved in the reaction vessel flows to the surrounding heat sink via a battery of thermocouples (thermopiles) which cover the vessel (Fig. 1). While the older Tian-Calvet type instruments use iron-constantan or cromel-constantan theromcouples, modern heat conduction calorimeters usually use more sensitive thermocouples with a higher Seebeck coefficient (**e**) (cf. Equation 2.15) such as platinum/ platinum-rhodium thermocouple (Hemminger and Höhne, 1984).

In the ideal case, the heat production rate (**P**) in the reaction vessel is related to the heat flux ($\frac{dq}{dt}$) through the thermopile wall and the heat capacity (**C**) of the reaction vessel plus leads, vessel contents, etc. according to Equation 2.1:

$$P = \frac{dq}{dt} + C\frac{dT_i}{dt} \qquad (2.1)$$

where ($\frac{dT_i}{dt}$) is the rate of temperature change of the reaction vessel (assuming thermal equilibrium with in the vessel). The heat flux ($\frac{dq}{dt}$) is proportional to the temperature difference ($\Delta T = T_i - T_e$) over the thermopile wall according to Equation 2.2. where $\mathbf{T_i}$ is the temperature of the reaction vessel (internal temperature) and $\mathbf{T_e}$ is the temperature of the heat sink (external temperature).

$$\frac{dq}{dt} = kn\Delta T \qquad (2.2)$$

where **k** is the thermal conductivity of a single thermocouple and **n** is the number of thermocouples.

The voltage (U) generated across the thermopile wall is related to the average temperature difference according to Equation 2.3:

$$U = ne\Delta T \qquad (2.3)$$

where **e** is the Seebeck coefficient.

Since the temperature of the heat sink (T_e) is constant, Equation 2.1 can be re-written as:

$$P = \frac{dq}{dt} + C\frac{d\Delta T}{dt} \qquad (2.4)$$

and by combination with Equations 2.2 and 2.3 as:

$$P = \epsilon(U + \tau(\frac{dU}{dt})) \qquad (2.5)$$

where,

$$\tau = \frac{C}{kn} \qquad (2.6)$$

is the time constant of the instrument. and

$$\epsilon = \frac{k}{e} \qquad (2.7)$$

is the calibration constant of the instrument which can be determined experimentally by electrical calibration. Equation 2.5 is often referred to as the **Tian equation**. The time constant, τ, is thus proportional to the heat capacity of the reaction vessel and inversely proportional to the thermal conductivity of the whole thermopile wall (K = kn). In practice,

$$\tau = \frac{C}{K} \qquad (2.8)$$

where K is the thermal conductivity of the whole thermopile wall plus vessel holders, leads,

etc. The time constant can be estimated from the half decay time of the thermopile signal ($\Delta t_{1/2}$) as the thermal power is turned off (cf. Fig. 2):

$$\Delta t_{1/2} = \ln 2 \tau \qquad (2.9)$$

Figure 2 shows the **power-time (P-t) curve** obtained from a heat conduction calorimeter measuring a constant heat production rate released between t_1 and t_2. As **U** approaches the steady-state value (**U_s**) asymptotically, being approximately equal to U_s (99.9%) after τ (Bäckman, 1991), Equation 2.5 is simplified to:

$$P = \epsilon U \qquad (2.10)$$

i.e., the power released is directly proportional to the voltage signal from the thermopile without taking in to account the time delay. The simple equation (2.10) is often the case (at least to a good approximation) in work with living systems where changes in heat production rate are slow (Wadsö, 1987).

If the differential term in Equation 2.5 i.e. $(\tau\ (\frac{dU}{dt}))$ is significant, the power must be corrected for the time-lag in order to obtain the actual heat production rate (**dynamic correction** [see e.g. Randzio and Suurkuusk, 1980]). Nowadays this can be performed automatically by use of a computer connected on-line to the calorimeter, but as yet it is rare to find that signals from thermopile heat conduction calorimeters are corrected to the true kinetic curves (Wadsö, 1987).

The total heat released (Q_t), the hatched area in Fig. 2, can be obtained from integration of Equation 2.5 over the time range between t_1 and t_3, where the latter is the time at which the voltage signal has returned to the baseline.

$$Q_t = \epsilon \int_{t_1}^{t_3} U dt + \epsilon \tau (U_3 - U_1) \qquad (2.11)$$

If the baseline is stable (i.e. $U_1 = U_3$), Equation 2.11 simplifies to:

$$Q_t = \epsilon \int_{t_1}^{t_3} U dt \qquad (2.12)$$

Since the area of the curve is directly proportional to Q_t, the latter can be calculated from the area (cm^2) of the recorder out put as:

$$A = \alpha Q_t \tag{2.13}$$

where α is the proportionality constant between area (A) and Q_t.

This shows that if the integration is extended to the time where the voltage signal has returned to the baseline, it is not necessary to know the time constant in order to determine Q_t.

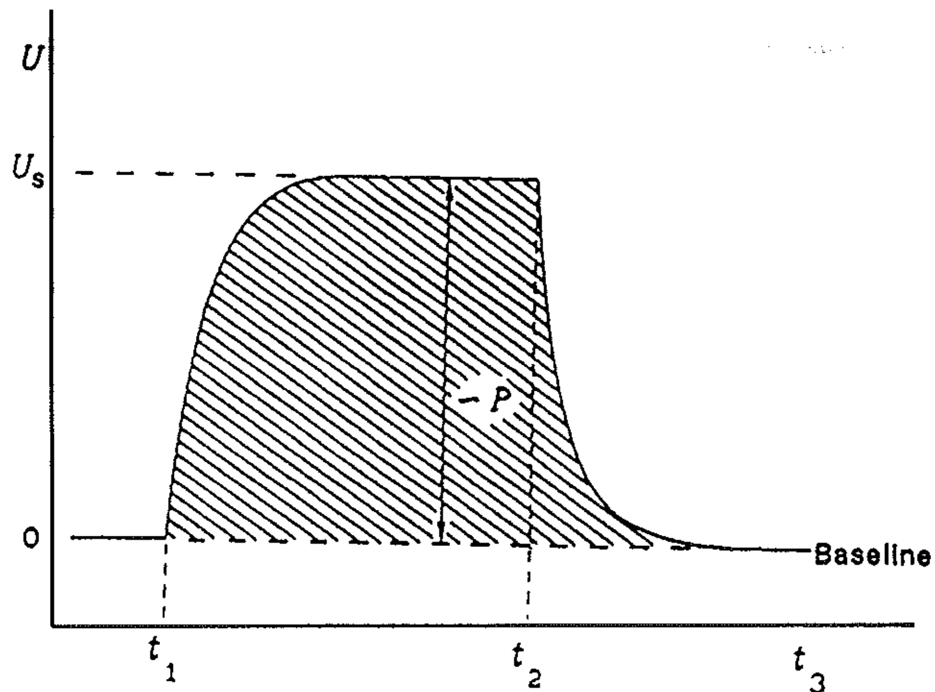


Fig. 2. Power-time curve obtained from a heat conduction calorimeter measuring a constant heat production rate (P) released in the interval t_1 to t_2. The hatched area corresponds to the total heat released (Q_t). Redrawn from Wadsö, 1987.

The sensitivity (S) of a heat conduction calorimeter can be expressed as a quotient between the values for the thermopile voltage and the power as:

$$S = \frac{U}{\left(\frac{dq}{dt}\right)} \tag{2.14}$$

which can be rewritten as (cf. Equations 2.2, 2.3, and 2.7):

$$S = \frac{e}{k} = \frac{1}{\epsilon} \qquad (2.15)$$

The sensitivity (S) is thus proportional to the Seebeck coefficient (e) and inversely proportional to the thermal conductivity per thermocouple (k).

2.1.3. Applications of calorimetry

Calorimeters in biology may be used as:

- **analytical tools** just to detect heat production and monitor it without any regard as to the quantity of heat evolved (Spink and Wadsö, 1976);

- **quantitative tools** to determine heat production and related thermodynamic figures of the system (Lamprecht, 1984).

2.1.3.1 Analytical tools

As analytical tools calorimeters are used to detect a possible heat flow between a sample and its surroundings and thus the possible existence of life processes in the probe. This principle is used in several areas of research such as:

a. Identification and/or characterization of microorganisms

In recent years a special field of analytical calorimetry grew up to some importance: the characterization and identification of microorganisms by their **'finger print'** like power-time curves. This field of research was started by Boling *et al.* (1973) and attracted much interest in the following years (Russel *et al.*, 1975a and 1975b).

Microorganisms growing in a complex medium exhibit a consecutive series of varying metabolic activities which are reflected in the rate of heat production. As metabolic behaviours differ in different microbes, structured power-time curves appear which are specific for a given organism. If one assures that the growth conditions (like pH, oxygen concentration, osmotic

pressure, etc.,) are well defined and properly chosen, highly reproducible and distinct P-t curves are obtained which can be used for the identification of organisms (Schaarschmidt and Lamprecht, 1976). In many cases identification can be done in as little as 3 hours (Newell, 1980), which is much quicker than with conventional methods.

b. Determination of contamination with and concentration of microorganisms

Between the field of calorimetry as an analytical tool detecting life and the field of quantitative calorimetry, there exists a domain in which calorimetry is used to detect a contamination with microbes and to quantitate them. One application is the control of milk daily delivered to a dairy industry, where flow instruments could be used in routine tests of contamination of large amounts of milk (Cliffe *et al.*, 1973).

In a similar line of experiments calorimetry was applied to follow the heat production and by this the microbial concentration in the effluent of sewage treatment plants (Tiefenbrunner, 1977). Calorimetry can also be used in clinical laboratories for the determination of contamination of samples of blood, urine, etc.

c. Influence of inhibitors on the heat production of microbial cultures.

Since calorimetry monitors metabolism of a culture it renders more information about the state of cells than other methods.

A constant heat production after the addition of a bacteriostatic drug demonstrates that growth is inhibited while metabolism occurs and the cells are still alive. If the heat production decreases, a bactericidal action is obtained. Thus calorimetry can be used in a semi-quantitative way to test the effect of various inhibitors or stimulators on the metabolism and growth of microorganisms. Again, such tests are by far quicker and sometimes even more sensitive than normal routine procedures (Beezer and Chowdhry, 1980), depending on the sensitivity of the calorimeter in use.

2.1.3.2 Quantitative tools

As quantitative tools calorimeters are used in studies ranging from pure chemical and biochemical reactions to heat generation by sub-cellular particles, microorganisms, tissues, organs, individuals, and even ecologic systems like the ant-hill (Lamprecht, 1983).

Systems from sub-cellular to the ecosystem level are investigated calorimetrically either in a mere analytical way to distinguish between rest and activity or in a quantitative way to determine levels of metabolism and compare them with data from indirect calorimetry such as polarography.

In the case of chemical/ biochemical reactions calorimetry is used to determine heats of hydrolysis, dilution, ionization, vaporization, denaturation, heats of transition in biopolymers, and for the determination of enzyme activities, evaluation of characteristic constants of enzymatic reaction (V_{max} and K_m), determination of substrate concentration in routine tests and the determination of binding constants, etc.

Calorimetry is thus a universal and reproducible, integrative, non-destructive, non-invasive, and non-specific method which determines the heat exchange accompanying every biological, chemical, or physical reactions. Consequently, it enables detecting life processes and monitoring metabolic activity without interfering with the system unlike biochemical tests, or in the absence of clear solutions unlike spectroscopy (Lamprecht, 1983).

Calorimetry can also be used as a quantitative tool for the investigation of drug-microbe interactions to establish dose-response curves. This is in addition to its use as an analytical tool just to detect whether or no a drug has an effect on a culture. The use of calorimetry, as an analytical and quantitative tool in microorganism-drug interactions, is a relatively recent innovation (Beezer and Chowdhry, 1981), used to overcome the problems encountered by the classical microbiological techniques in testing the effect of antimicrobial agents such as propolis, and to obtain better results.

2.2 PROPOLIS

The word **propolis**, as defined by Merriam Webster's Seventh New Collegiate Dictionary (p. 683), has originated from two Greek roots: *pro*, which means for or in defence, and, *polis* which means town or city. Literally, therefore, propolis means for or in defence of the city/town. All sources consulted seem to agree that the word refers to its use in partially closing the entrance or gateway to the bee commune or city (Iannuzi, 1983).

Propolis (bee-glue) is a resinous natural product of honey bees like wax and honey and accumulates in the bee hives. There are many investigations on the origin of propolis. It is generally accepted that bees collect propolis from resinous tree buds and a tremendous amount of trees have been proposed as sources of propolis (Crane, 1988). But only in few cases chemical analysis has been performed in order to confirm these proposals (Bankova *et al.*, 1995). Almost always poplar buds appeared to be the sources of propolis in temperate zones, especially *Poplus nigra L.* (Greenaway *et al.*, 1987; Lavie, 1976; Papay *et al.*, 1986). Trees of secondary importance include the birch, oak, alder, willow, and hazel in Europe; the *Acacia karoo* tree in South Africa; the grass tree *Xanthorrhoea pressi* and *X. australis* in Australia; *Plumeria accuminata*, *P. acustifolia* and *Psidium guajava* in North America (Ghisalberti, 1979). However, nothing is known on the plant source of propolis in the tropics (Tomas-Barberan *et al.*, 1993). Investigations on propolis from Venezuela (Tomas-Barberan *et al.*, 1993) and Brazil (Aga *et al.*, 1994) showed absence of polyphenols from poplars which are chief constituents of temperate propolis. Different propolis samples differ substantially in their chemical composition, depending on the geographical location of the collection site (Ghisalberti, 1979). This variation in the chemical composition of propolis is inevitable since different geographical localities possess various plant compositions and hence bees use different plants as sources of propolis. Variation may occur in the chemical composition of propolis collected from the same geographical locality but different bee hives, a few distance apart. This is because different bee communities may not have equal access to some plants as sources of propolis. Although the chemical composition of propolis shows a high variation from one geographical locality to another, the basic makeup is usually maintained as 55% resin, 30% wax, 10% essential oils and 5% pollen (Ghisalberti *et al.*, 1978). Resin comprises varying amounts of many complex chemicals from plants and glandular secretions of worker bees that are extractable in 70% alcohol. It can therefore be asserted that the chemical

composition of propolis is very complex; and more than 160 constituents have been identified so far from propolis of different geographical origins (Bankova *et al.*, 1995). The type and amount of various chemicals present in different propolis samples may show variation based on the abundance of plant varieties which can be used as sources of propolis in different localities. Thus, those propolis samples that are collected from an area of high plant diversity, may possess higher variety of chemicals than those collected from lower plant diversity areas. Propolis varies in colour from light brown to a dark chestnut, depending on its source and age, and has a strong aroma. When new, at the normal temperature of hives, it is very sticky. Below 15°C it becomes quite hard and below 5°C it is brittle (Ghisalberti, 1979).

Propolis is gathered by honey bees from leaf buds, breaks in the bark of trees and coating of pollen grains. Worker bees then add glandular secretions, such as enzymes and wax to it in the bee hive (Greenaway *et al.*, 1987). Except for the incorporation of glandular secretions, no change in the chemical composition of propolis, directly obtained from plants by the honey bees, occurs (Ghisalberti, 1979). Propolis was developed over millions of years of evolution as a medicinal agent by the trees themselves to protect infection of injured tree parts, retard bud development while frost, and protect pollen from being infected by microbes ensuring reproduction and perpetuation of the species (Ogren, 1990).

Honey bees use propolis to block up any opening in the hive and to varnish all the inside surfaces of the hive, not only the wood work and the frames of the bee hive, but also the wax cells themselves (Ghisalberti, 1979). The presence of propolis as a thin layer on the inner wall of the bee hive prevents loss of moisture and hence renders the inside of the hive moist and warm, a condition needed by the developing brood (Möbus, 1972). The moist and warm conditions and the occurrence of tens of thousands of bees crowded in to a small area in the hive provides conditions for all kinds of bacteria and mould to flourish. The presence of propolis varnish, however, prevents these occurrences as it does on tree parts mentioned above. It has been suggested (Derevici *et al.*, 1964) that propolis is in fact responsible for the lower incidence of bacteria and mould within the hive than in the atmosphere outside. The volatile constituents of propolis reduce the aeroflora within the apiary (Derevici *et al.*, 1965). Apart from its use as a varnish and filler of cracks and holes it is used to make the entrance of the hive weathertight and easier to defend. Bees instinctively fill the need to use propolis to seal up cracks and holes in the hives to keep out the chilly winds (Ogren, 1990). For this

reason, bees in cooler regions are prone to propolize the hive a great deal more than those in warmer regions.

A mixture of propolis and bees wax is much stronger than bees wax itself (Thomson, 1990). Hence bees use the mixture to make the borders of combs stronger where they are attached to the wooden framework of the hive. It is also used to repair combs in cases of cracking.

When small intruders, such as wasps, enter a bee hive they are killed soon and thrown out by the bees. However, when an intruder is too large to be moved out (for instance a large moth or a mouse) bees first kill it and then encase the carcass in propolis so that it does not rot and cause a bad smell but slowly mummifies. The embalming of intruders may serve to contain putrefaction, and thus the spread of infection and disease (Ghisalberti, 1979).

The production of propolis at a large scale can be achieved by several methods which stimulate the bees to collect more and more propolis. These include: (i) removal of propolis from the entrance of the hive so that they collect a tremendous amount of propolis to make the entrance narrow; (ii) putting some foreign materials, simulating an intruder into the bee hive, so that they embalm it with propolis; (iii) making cracking in the internal wall of the hive and/or the comb so that they repair it with propolis. Consequently bees gather much amount of propolis which can be collected and used by human beings for different purposes.

The medicinal value of propolis to the honey bees is equally beneficial to man kind. Propolis has been used in folk medicine since the time of the Pharaohs (Lamprecht, 1994). There is evidence that in prehistoric times, surgery was made possible by the use of propolis as an antiseptic (Thomson, 1990). Nowadays, propolis has attracted attention as a natural cure as well as for clinical applications due to the development of drug resistance by most pathogenic microbes and the wide spread occurrence of disfunction of the immune system (Dimov *et al.*, 1991). Immunomodulation through natural or synthetic substances may be considered an alternative for the prevention and cure of infectious diseases (Azuma and Jolles, 1987; Hadden *et al.*, 1989; Toshkov *et al.*, 1989). Propolis possesses versatile biological activities including antibacterial, antiviral, fungicidal, immunostimulating, hypotensive, cytostatic (Ghisalberti, 1979), antiinflammatory (Wang *et al.*, 1993) and antiulcer (Scheller *et al.*, 1989). The number of different preparations from raw propolis is, therefore, as complex as the diversity of described medical applications for e.g. dental problems, dermatology or infections of the respiratory duct. Most fields of application still base on the excellent wound

healing and antiinflammatory properties of propolis (Volpert and Elstner, 1996). Nowadays propolis is used in foods and beverages intended to maintain or improve human health due to its beneficial effects and the 'back to nature' trend (Dobrowolski *et al.*, 1991).

Though the antibacterial activities of propolis and some of its constituent compounds have widely been reported, its mechanism of action (bactericidal/ bacteriostatic) have not yet been clearly understood. Therefore, it is important to investigate its mode of action by the use of a calorimeter. Propolis is widely used for different medical applications in Asia, Europe and America. Up to now no reports exist on the antibacterial activity of propolis in Ethiopia. One of the objectives of this work was therefore to test the antibacterial activity of Ethiopian propolis collected from the Holleta Bee Research Centre and compare its effect with propolis of different geographical origins.

If propolis from poor countries such as ours is found effective against pathogenic microorganisms it can be recommended for use instead of antibiotics since the poor may not afford to buy the latter. The use of propolis and other natural preparations as antimicrobial agents may help to alleviate the economic constraints of the poor to buy drugs. In addition, in some remote rural areas where access of the people to medicine is highly limited it may serve, apart from folk medicine, as the sole/ main medicinal agent since it is found in most places where bees are found.

Several clinical findings point to the fact that propolis may be more effective against pathogenic microorganisms than conventional medications, with the added advantage that it causes minor side-effects (Azevedo *et al.*, 1986), as is true of many natural preparations. Thus, it is of advantage to use natural preparations, such as propolis, not only by the poor but also by the wealthiest nations.

Since propolis is a coloured mixture, it is impossible to investigate its antibacterial activity spectrophotometrically as the colour interferes with absorbance. However, since calorimetric results are not affected by colour, calorimetry is the best method to investigate action of propolis on bacterial growth and metabolism.

2.3 Objectives of this work

The main objectives of the present work are summarized as follows:

a. To establish a P-t curve of the organism in the absence of inhibitors and to compare it with results simultaneously obtained from polarography and spectrophotometry in order to see if the P-t curve is a true picture of the metabolic events in the fermenter.

b. To investigate if inoculum size, which is a serious problem in classical microbiological techniques, affects calorimetric results.

c. To compare the sensitivity of the calorimetric method with the agar well diffusion assay based on the lowest concentration of different propolis samples exhibiting inhibition and/ or killing (MIC).

d. To incorporate propolis at different phases of the P-t curve and to observe the kinetics of response.

e. To compare the antibacterial activity of different propolis by incorporating them at the mid exponential phase of the P-t curve.

f. To investigate the mode of action of propolis and to compare this with that of a known antibiotic, ampicillin.

g. To separate the water soluble and insoluble components of propolis and to investigate the antibacterial activities of both and to compare their effects with that of EEP.

h. To compare the antibacterial activity of different propolis by the agar well diffusion assay method.

3. MATERIALS AND METHODS

3.1 BIOLOGICAL MATERIAL AND GROWTH CONDITIONS

All experiments were performed with the bacterium *Micrococcus luteus*, (strain 348, ATCC 9341). The organism was a kind gift of Prof. Dr. Gunda Kraepelin, Technical University of Berlin Institute of Microbiology. *M. luteus* is Gram positive and cells are irregularly arranged.

The growth medium was composed of peptone (10 g L^{-1}), $MgSO_4.7H_2O$ (0.247 g L^{-1}), and NaCl (5 g L^{-1}) in deionized water. Solid growth medium was prepared by incorporating 15 g L^{-1} agar to the liquid medium mentioned. The pH of the growth medium was adjusted to 7.0 by using 1N NaOH and/or 1N HCl prior to sterilization (15 minutes at 121 °C). Stock cultures were kept on solid slants at 4 °C. One day before each experiment, cells were inoculated in to a 50 mL Erlenmeyer flask containing 20 mL liquid medium and incubated at 30 °C on a rotary shaker at a stirring rate of 125 rotations per minute (rpm).

Though the organism grows at 30 °C to transfer it from stock to experimental conditions, it was important to investigate the temperature at which it shows optimal growth. This was done spectrophotometrically as follows: the culture grown for 24 hours at 30 °C was diluted with the liquid growth medium to make an initial culture of optical density (OD) = 0.1. Optical density was measured using a spectrophotometer (type UV-120-01 Shimadzu Kyoto) at 646 nm. The culture (30 mL) with an OD of 0.1 was put in a 50 mL culture vessel and incubated in a water bath of predetermined temperature by stirring with a magnetic stirrer at the bottom. Optical density measurements were done for 18 hours starting at the time of inoculation (zero hour) in an interval of 1 hour. The temperature of incubation ranged from 5 to 50 °C with an interval of 5 °C. Those water baths whose temperature had to be below room temperature were put in a cold room (4 °C).

According to Lambert's Law cell number and OD have direct relationship up to an OD value of 0.6-0.8. Hence, when the latter approached these values reading was done after a 1:2 dilution with the media used for growth.

The OD values obtained for each temperature were plotted against time (h) on a semilogarithmic graph paper. The generation time was determined from the exact doubling

of OD in the exponential phase of growth. For those temperature values where doubling of OD was not achieved within the given time, but a slight increase was observed, the generation time was extrapolated from the percentage increase of OD during the incubation period where the maximum growth rate was observed.

For experiments involving cell number, cell counting for each experiment is generally laborious and could introduce experimental error. To alleviate these problems the relationship between OD and cell number was established by using cells grown at 30 °C. Cell suspensions were taken at intervals of 1-2 hours and OD determined at 646 nm. A volume of the suspension was then put on a cell counting chamber (Helber/ Germany) of quadrates 2 x 10^{-3} cm deep and 2.5 x 10^{-5} cm^2 wide. For each sample 112 quadrates were counted and each of these were done in triplicates. The average cell number mL^{-1} of the three counts was taken.

3.2 EXTRACTION OF PROPOLIS

Propolis samples of different geographical origin were used. These include propolis from Germany (prop1) and Uraguay (prop2) both obtained as a gift from the Pharmaceutical Institute of Free University of Berlin, Ethiopian propolis (prop3) collected from Holleta Bee Research Centre and Aagaard propolis (10% propolis tincture in 60% ethanol)(NF Börner/ Germany) of German origin. Aagaard propolis is on market as a medicinal agent for the treatment of upper respiratory tract infections.

Each propolis sample, except the tincture, were homogenized by a coffee mill (type MZ_{22} Moulinex/ France). The ground mist was suspended in 70% methanol (Greenaway *et al.*, 1990). Volume of methanol needed was in such a way that 10% (w/v) of the mist was achieved for effective extraction (Strehl *et al.*, 1994). The suspension was agitated and added into a Rotavapour (Heidolph Rotations Verdampfer W-micro, Manheim/ Germany) extraction apparatus and was mixed continuously for 2 hours at 60 °C and at a rate of 200 rpm. After the allocated time the suspension was suction filtered using a filter paper, Selecta Filter NR 595 1/2, (Volpert and Elstner, 1993). The filtrate was put on a tray and placed in an oven at 40 °C to evaporate the solvent. Complete loss of moisture was confirmed by a day to day inspection for weight loss using a sensitive balance (Fleinwaage type 414/13, Sauter D-7470 Ebingen, Germany) until no weight loss was observed for three continuous weigh-ups, which was achieved within 15 days. The yield of extraction was approximately 21% (w/w) for all propolis

samples used. The powdered form of the extract was dissolved in 60% ethanol to make a 10% (w/v) stock solution and stored in a refrigerator for later use.

The ethanol solution of extract of propolis (EEP) possesses both water soluble and insoluble components. Addition of this propolis solution to the growth medium resulted in the formation of white precipitate, a problem mentioned previously too (Dimov *et al.*, 1992 and Matsuno, 1995). To separate the water insoluble components of propolis 1 mL of the 10% stock solution was mixed with 9 mL distilled water making the water insoluble components to precipitate out. The precipitate was separated by centrifugation at 4,500xg for 50 minutes (Volpert and Elstner, 1993). The precipitate fully entered in to solution with 60% ethanol and maintained the colour of the original propolis solution. The water soluble component however, had a colour of whitish-grey for all propolis. The water soluble and insoluble components, both in solution, were dried in an oven at 40 °C. Complete loss of moisture was confirmed as above. The dry powder was then weighed and dissolved in the appropriate solvent (distilled H_2O or ethanol) to make a 10% stock solution. The stock was then used in calorimetry and agar well diffusion assay side by side with the unprecipitated EEP. Comparison, by calorimetry, of activity of the water soluble and insoluble components with EEP and among themselves was done at a concentration of 1 mg mL^{-1} (since some components do not have activity below this concentration), and using their minimal inhibitory concentrations (MIC).

3.3 CALORIMETRY

A flow calorimeter (type 10700-1, LKB/Bromma, Sweden) with a flow-through cell of volume 0.587 mL was used for the entire calorimetric work. Having established the optimum temperature of growth of *M. luteus*, the working temperature of the calorimeter was set at 30 °C by direct electrical heating. The calorimeter uses a prethermostat water bath set at 22 °C. The calorimeter was connected to a separate fermenter in a water bath controlled at 30 °C. The connection was made by a peristaltic pump (LKB/ Pharmacia, Sweden) and a teflon tube of $\phi = 0.1$ cm. The tube connection between the calorimeter and the fermenter could house different electrodes for the simultaneous determination of heat production and others such as oxygen consumption and pH value. In the present work an oxygen electrode (Eschweiler/ Kiel, Germany) was incorporated in the flow line directly behind the calorimeter for the determination of oxygen concentration in the out flow line.

To satisfy the requirement for both a high flow rate (to avoid settling of cells and exhaustion of substrates during the residence time in the tubing system) and for a slow flow rate (to allow sufficient temperature equilibration and to avoid creating heat by friction), a pumping rate of 100 mL h^{-1} was used for investigations with strong aeration (Motzkus *et al.*, 1993). At this pumping rate it took a sample 1.42 min to reach the calorimetric cell and 3 min to return back to the fermenter.

The calorimetric signal was recorded by a two channel recorder (BD5, Kipp and Zonnen) at a full scale deflection of 50 μV and a chart speed of 0.02 cm min^{-1}.

3.3.1 Sterilization of the flow line

Since *M. luteus* may grow on the inner wall of the vessel, intensive cleaning was necessary before and after each experiment. A solution of 10% H_2O_2 and 2% H_2SO_4 in distilled water (Motzkus *et al.*, 1993) was pumped through the flow line and allowed to act for at least 20 min. After the allocated time for sterilization the flow line was washed by a sterile solution of potassium phosphate buffer of pH 7.0 for at least 30 min. Then the sterile growth medium was pumped through the flow line until a stable baseline was obtained, which was achieved with in an hour. The cell suspension was then added to establish a P-t curve.

Incorporation of propolis to the fermenter and pumping it in the flow line creates a problem in latter cleaning of the line. Since the water insoluble components of propolis precipitate on the inner wall of the vessel and can not be removed during sterilization and washing by the solution mentioned above a solution of 10% H_2O_2 and 2% H_2SO_4 in 60% ethanol was used. In this solution, ethanol dissolves the water insoluble components of propolis which precipitates on the inner wall while the other components lyse bacterial cells so that the line is clean and sterile. Then the routine washing with the buffer follows.

3.3.2 Calibration of the calorimeter

Calibration of the calorimeter to determine its sensitivity (S) at the experimental pumping rate was done by directly sending electrical current of known quantity via the calibration heater. The heater has a resistance of 49.479 Ohm. Before calibration the flow line was sterilized. Since sensitivity is dependent on the pumping rate (Poore and Beezer, 1983),

calibration was done by pumping a sterile potassium phosphate buffer of pH 7.0 at a rate of 100 mL h^{-1}, a rate used throughout this work.

The current supplied via the calibration heater creates a voltage difference across the thermopile wall. The amount of thermopile voltage generated was represented by the recorder deflection on the y-axis. The chart speed was adjusted at 0.1 cm min^{-1}. Since the recorder full scale deflection was 50 μV/ 20 cm, a 1 cm deflection on the y-axis represents 2.5 μV. The recorder deflection (cm) on the y-axis was then multiplied by 2.5 to find the voltage (**U**) generated. For the different current values supplied the power, **P**, was calculated from $\mathbf{P = RI^2}$, where **R** = resistance of the calibration heater (ohm) and **I** = calibration current (ampere). Plot of the voltage signal generated **U** (μV) as a function of power supplied **P** (mW) produces a regression line. The slope of the regression line is the sensitivity, **S, (μV/mW) of the** calorimeter.

3.3.3 Calorimetric cultivation of cells and effect of EEP

Having established a stable baseline by pumping the sterile growth medium through the calorimeter, a cell suspension that has grown for the last 24 hours at 30 °C was used as an inoculum. Before starting the experiment with propolis it was important to establish the metabolic behaviour of the organism and look if the inoculum density affects the shape of the P-t curve and total heat production. For this end variation of the inoculum density at the beginning of the calorimetric growth was achieved by adding 25, 50, 100, 200, 400, 600, and 800 μL of cell suspension to the fermenter (50 mL culture vessel) containing 30 mL growth media. From the relationship between OD and cell number mL^{-1}, Fig. 4, the above inoculum densities correspond to 0.078, 0.086, 0.14, 0.34, 0.92, 1.4, and 2.4 ($x10^7$) cells mL^{-1}. The culture was highly aerated by stirring from bottom using a magnetic stirrer. The calorimeter was run for *ca* 25 h to establish a P-t curve.

In order to establish the relationship between heat production rate and OD or cell number mL^{-1} and pH value, 1 mL sample was taken at the outlet of the flow line. Optical density was measured at 646 nm and the same sample was used to measure pH using (pH-meter 761 calimatic, Knick Micro Prozessor Gmbh/ Berlin). Samples were taken at an interval of 1 hour starting at 10 minutes after inoculation (time needed for the cell suspension to be

uniformly distributed). Optical density and other parameters were determined at the peak of the P-t curve to see what parameter changes drastically at this point.

For investigations with propolis an inoculum volume of 500 µL/ 30 mL media was used to achieve a cell density of *ca* 9.8 x 10^6 cells mL^{-1} as an initial culture. The cells were allowed to grow in the flow calorimetric system and a P-t curve was established. To see how the P-t curve responds to antibacterial agents at different phases of growth, increasing concentrations of tincture were added at the late lag phase/ beginning of the exponential phase, mid exponential phase, immediately after the main peak of the P-t curve, and after the fall of the P-t curve to a minimum value. For comparison of the effect of different propolis samples on the P-t curve increasing concentrations of EEP for each propolis were added at the mid exponential phase of the P-t (Beezer and Chowdhry, 1980), before the first peak. The exponential phase where EEP was incorporated corresponds to a cell density of *ca* 5.1 x 10^8 cells mL^{-1} and heat production rate of 0.54 mW mL^{-1}.

To compare the effect of propolis with known antibiotics, sodium salt of ampicillin (amino benzyl penicillin sodium salt) (Biochemica/ Fluka, Germany) was used. Ampicillin is known to be active against gram-positive and gram-negative bacteria. After addition of an antibacterial agent the calorimeter was run for 10 to 15 hours. The solvents, 60% ethanol for propolis and distilled water for ampicillin, were used as controls to see if they have any effect on the P-t curve.

3.3.4 Treatment of the P-t curve

The areas under the obtained calorimetric P-t curves were determined by means of an electronic planimeter (Digikon, Kontron/ Munich). The total heat (Q_t) produced during the experiment was calculated from the area of the P-t curve according to Equation 2.13, i.e.,

$$A = \alpha Q_t$$

where A is the area under the P-t curve, Q_t is the total heat produced during the growth period, and α is the proportionality constant between the two. The proportionality constant can be calculated as follows: an area of 1 cm^2 on the curve represents 1 cm on the x-axis and 1 cm on the y-axis. Since the recorder is adjusted at a full scale deflection of 50µV (i.e. 50 µV/ 20 cm), every 1 cm length on the y-axis represents 2.5 µV. On the x-axis the recorder is adjusted at a

chart speed of 0.2 mm min^{-1} (= 50 min cm^{1}). Therefore a 1 cm^2 area under the P-t curve represents

$$2.5\ \mu V \times 50\ min = 125\ \mu V\ min$$

Since the calorimeter has a sensitivity (S) of 54.12μV mW^{-1}, taking the reverse of this gives

$$\epsilon = 18.48\ \mu W\ \mu V^{-1}.$$

Substituting this value, i.e., 1 μV = 18.48 μW in to the relationship 1 cm^2 = 125 μV min, gives a value of

$$1\ cm^2 = 138.6\ mW\ sec = 138.6\ mJ$$

From the equation $A = \alpha Q_t$; for an area of 1 cm^2 :

$$1\ cm^2 = \alpha \times 138.6\ mJ$$

dividing both sides by 138.6 mJ gives

$$\boldsymbol{\alpha = 7.23 \times 10^{-3}\ cm^2\ mJ^{-1}}$$

Therefore, the area (cm^2) under the P-t curve, determined by planimetry, divided by α gives the total heat produced in Joule. Since volume of the calorimetric cell is 0.578 mL, Q_t obtained here is that per 0.587 mL. This value was converted to J mL^{-1} values.

Not only areas of the P-t curve but also the curves themselves were directly transferred from the recorder out put to a computer screen by using the digitalizer (planimeter) fitted to a personal computer (PC). The computer program for the transfer of data from the digitalizer to PC was written and licensed by the Institute for Biophysics of the Free University of Berlin.

For the extrapolation of data from the curve and to transfer it to PC the appropriate scale factors were used. It was found out that 1 cm on the y-axis represents 2.5 μV x 18.48 μW μV^{-1} = 0.046 mW. Since this is the power out put for a volume of 0.587 mL it is equal to 0.078 mW mL^{-1}. Therefore, the displacement (cm) of the curve at any point on the y-axis from the baseline is multiplied by 0.078 mW mL^{-1} cm^{-1} to find the power output in mW mL^{-1}. Finally, the curve was established based on power (mW mL^{-1}) as a function of time (h).

3.4 POLAROGRAPHIC OXYGEN SENSORS (POS)

It was suggested (Hölzel *et al.*, 1994) that oxygen becomes a limiting factor in the flow line at higher cell densities, elevated substrate concentration and/or reduced pumping rates. As a result the calorimetric signal may no longer be a true picture of the metabolic events in

the fermenter. Due to this a POS (type EaK1 Eschweiler/ Kiel, Germany) equipped with a magnetic stirrer was incorporated in the flow line behind the calorimeter. The signal from the POS was recorded by a two channel recorder (type LS4 Linseis, Gmbh/ Germany) at a full scale deflection of 20 mV and a chart speed of 10 mm h^{-1}.

The oxygen consumption rate of the cell suspension was measured in parallel with the flow calorimetric experiments by a batch oxygen electrode (Bachofer/ Reutlingen, Germany) equipped with a magnetic stirrer. This was mainly done to see if the fall of the P-t curve to the baseline after the peak was due to oxygen limitation in the flow line or to the entrance of the cells in the stationary phase of growth because of other constraints such as depletion of nutrients and/ or accumulation of metabolic wastes in the fermenter in addition to the depletion of oxygen in the flow line due to high cell density. If the oxygen consumption rate after the peak remains constant at a certain higher value it shows that the cells have entered the stationary phase of growth and hence the P-t curve would have remained at a higher value had it not been for the limitation of oxygen in the flow line. If the fall of the P-t curve to the baseline is due to other factors such as limitation of nutrients or accumulation of metabolic wastes the oxygen consumption rate also falls off since there is no significant metabolism. The batch oxygen electrode then helps to check if other factors, in addition to oxygen limitation in the flow line are involved for the drop of the P-t curve after the peak.

All polarographic experiments were done at 30 °C by circulating a thermostated water through the outer compartment of the electrode.

For rate determination with the batch oxygen electrode, aliquotes of 1 mL were transferred to the electrode at given times of the growth curve from the outlet of the flow line. The suspension was then aerated by pumping an atmospheric air using a pump (Rena 101/ Germany) for 5 min. Aeration was important especially at higher cell densities after the peak of the P-t curve where the oxygen concentration in the suspension transferred to the electrode was almost zero. After aeration the electrode was plugged excluding the entrance of air and the experiment was run for 5 min. The signal from POS was recorded by a two channel recorder (Servogor XY 743, Goerz/ Austria) at a full scale deflection of 20 mV and chart speed of 3 cm min^{-1}.

Polarographic experiments for treatments with EEP were not possible due to precipitation of the water insoluble components on the surface of the membrane and hence blockage of the diffusion of oxygen to the electrode. In addition to this, for the POS

incorporated in the flow line, the tubing system in the sensor compartment of the POS was completely blocked due to continuous precipitation of the water insoluble components regardless of stirring; consequently pumping stops. Due to these problems antibacterial activity of propolis was not investigated polarographically.

Calibration of the POS was done by using an air saturated water and a 3% solution of $Na_2S_2O_4$. Air saturated water, achieved by bubbling atmospheric air through distilled water for *ca* 20 min, possesses 237 μ mol L^{-1} dissolved oxygen at 30 °C (Forstner and Gnaiger, 1983). The 3% $Na_2S_2O_4$ solution devoids water of any dissolved oxygen and hence a zero point achieved (Hitchman, 1983). For calibration the air saturated water was first added to the electrode and kept air-tight until a stable saturation line was produced by the recorder, which was achieved with in 2-3 min. Then the water was removed and the zero point calibrating solution was added. The electrode compartment was kept air-tight until a stable baseline was achieved, which took *ca* 4-5 min.

The saturation value, 237 μ mol L^{-1}, divided by the recorder deflection (cm) from baseline to oxygen saturation gave a value of oxygen concentration for a unit length (cm) of recorder deflection, in μ mol L^{-1} cm^{-1}. The concentration of oxygen (μ mol L^{-1}) in the flow line at any point along the recorder out put was determined by measuring the recorder deflection (cm) from the baseline and multiplying it by the oxygen concentration per unit length.

Rate of oxygen consumption by the batch oxygen electrode was determined by multiplying slope of the curve by the calibration factor. Since the chart speed was 3 cm min^{-1} (= 20 sec cm^{-1}), dividing the value of oxygen concentration per unit length of recorder deflection by 20 sec cm^{-1} gave the calibration factor in μ mol $L^{-1}sec^{-1}$. This unit is the unit used for the expression of rate of oxygen consumption.

3.5 AGAR WELL DIFFUSION ASSAY

In addition to calorimetry, the antibacterial activity of propolis was investigated by the agar well diffusion assay method. A sterile growth medium was poured in to sterile petri-dishes on labelled surface and solidified to make a 3 mm thick layer. A cell suspension that has grown for the last 24 hours was diluted with the liquid growth medium to make an inoculum of OD 0.8 at 646 nm. To achieve a lawn of growth of cells 0.5 mL of the inoculum was inoculated on the plate and spread by using a flame sterilized and cooled Z-shaped glass

rod. After an hour wells were cut in the agar with a flame sterilized and cooled cork borer of $\phi = 8$ mm and the agar plugs were removed with a sterile needle. On each plate four wells were made so that each sample could be done in quadruplicates. In the case of higher concentrations of ampicillin however, a single well was made at the centre of the plate due to the large diameter of the clear zone. In all cases each concentration was done in quadruplicates. Each well was then filled with 100 μL of a given concentration. Blanks of 60% ethanol and distilled water were used for propolis and ampicillin respectively. The latter blank was also useful for the water soluble components of propolis.

The plates were incubated for 48 hours. The clear zones were displayed on a Microfiche screen (Robot/ Germany) and magnified 9.8 times. Each clear zone including area of the agar well was traced on a transparent paper. Areas of the clear zone and the well were determined by the electronic planimeter. Subtracting area of the well (which was equal in all cases) from the total area gave area of the clear zone. Calculating radius of the clear zone from the area and dividing it by the magnification factor (9.8) gave the mean radius of the clear zone on the plate. Antibacterial activity was then expressed as the radius of the clear zone (mm).

4. RESULTS

4.1 DETERMINATION OF OPTIMUM GROWTH TEMPERATURE

The influence of temperature on the growth of *M. luteus* was represented by its effect on the generation time (t_g). Plot of t_g versus temperature (Fig. 3) showed the pattern of effect of temperature on the growth and thus the metabolism of the organism.

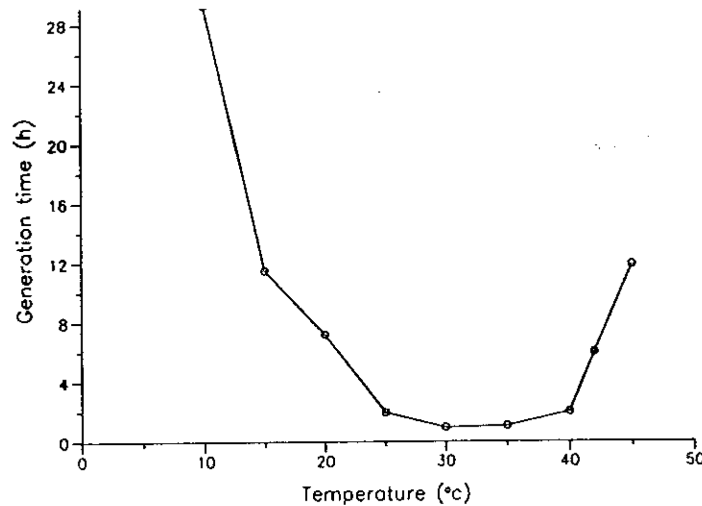


Fig. 3. Effect of temperature (°C) on the generation time (h) of *M.luteus*.

At temperatures below 10 and above 45 °C no growth was observed even after incubating the culture for 48 h showing that the growth temperature lies in between these temperature values. Above 45 °C slight decrease in OD was observed during the incubation period.

The organism showed optimal growth between 25 and 40 °C with the minimum t_g at 30 °C. Below the lower value of the optimum range (25 C) temperature coefficient of the growth rate first failed off with a Q_{10} of 4 to 5 i.e. a four to five fold decrease in growth rate per 10 °C drop in temperature. It then declined more rapidly giving rise to a fairly well defined minimal growth temperature of 10 °C. Above the upper value of the optimum range (40 °C) the growth rate decreased steeply with increasing temperature giving rise to the maximum growth temperature, 45 °C, not too far from the optimum range.

4.2 CELL NUMBER VERSUS OPTICAL DENSITY

Optical density of the culture was determined and its cell number mL^{-1} counted. For a highly turbid culture, OD was determined after dilution. Plot of OD as a function of cell number mL^{-1} (Fig. 4) showed the relationship between the two with slope of the regression line equal to 0.394 and index of correlation, (R^2) = 0.995. The index of correlation indicates that the two characters show a good correlation.

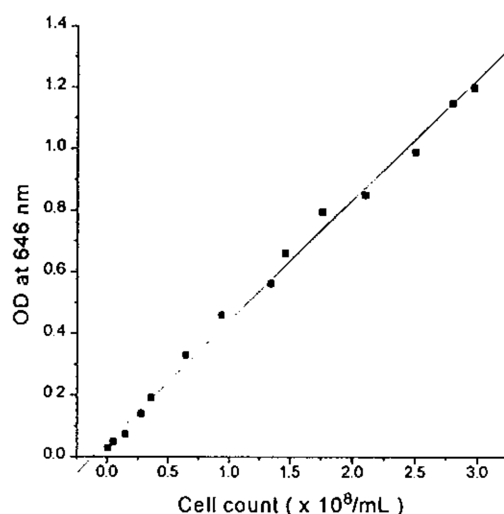


Fig. 4. Plot of OD (at 646 nm) versus cell number mL^{-1} for *M. luteus* grown at 30 °C. Equation of the regression line is **Y = 0.0435 + 0.39 * X, and R^2 = 0.995**

4.3 PROPOLIS EXTRACTION

Extraction of propolis in 70% methanol resulted in a yield of *ca* 21% (w/w) for all propolis samples used. This value was comparable with the yield of 23% (w/w) obtained by Matsuno (1995) for propolis samples from Brazil.

Precipitation of the water insoluble constituents of EEP by the addition of 9 mL distilled water in to 1 mL EEP showed that propolis possesses both water soluble and insoluble components which are soluble in alcohol.

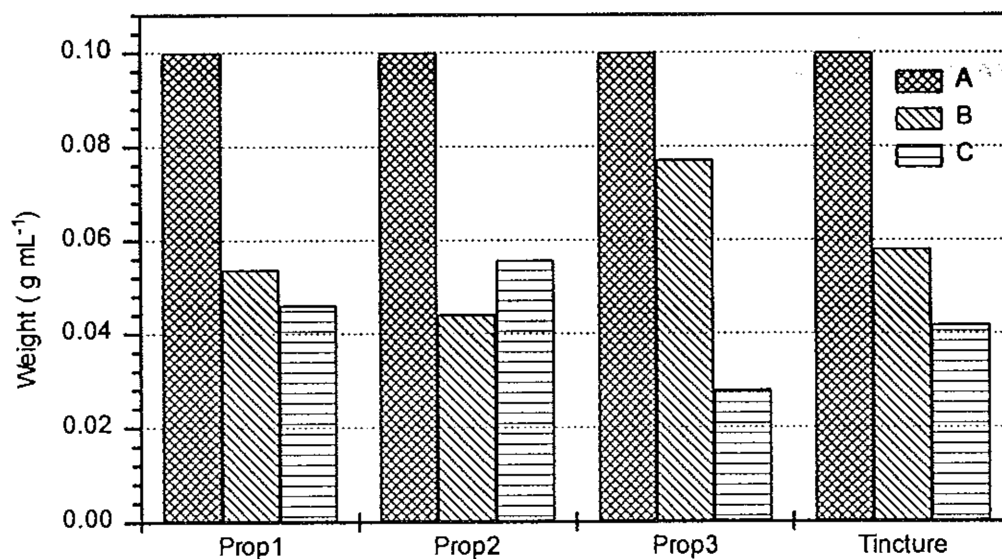


Fig. 5. Weight (g mL^{-1}) of the different components of EEP obtained by taking 1 mL of 10% EEP and mixing with 9 mL distilled water to precipitate out the water insoluble components. For all propolis samples, 0.1g EEP (A) possesses water insoluble components (B) and Water soluble components (C).

Though all propolis samples showed an almost equal yield (w/w) during extraction by 70% methanol they have different percentages of water soluble and insoluble components. Prop3, unlike the others, showed a very low proportion of water soluble components (Fig. 5).

4.4 CALORIMETRY

4.4.1 Electrical calibration

Electrical calibration of the calorimeter was done by sending an electrical current of known quantity through the calibration heater. Since the resistance (R) of the heater is known, the power supplied was calculated based on the equation **P = RI²**, by taking square of the current supplied (Table 1). Dividing the voltage signal generated by the power supplied for each current value gives the sensitivity, **S**, (μV/mW).

Table 1. Result of electrical calibration of the flow calorimeter by a heater of resistance, R = 49.479 ohm and at a pumping rate of 100 mL h^{-1}. **I**: current supplied; **I^2**: square of the current supplied; **P**: power supplied (= RI^2); **d**: recorder deflection; **ΔU**: voltage generated (d cm x 2.5 μV cm^{-1}) and S: sensitivity (ΔU/P).

I (mA)	I^2 (mA^2)	P (mW)	d (cm)	ΔU (μV)	S (μV mW^{-1})
1.231	1.515	0.075	1.65	4.125	55.00
1.741	3.031	0.150	3.25	8.125	54.17
2.130	4.537	0.225	4.85	12.125	53.89
2.462	6.061	0.300	6.55	16.375	54.58
2.753	7.579	0.375	8.15	20.375	54.33
3.016	9.096	0.450	9.90	24.750	55.00
3.257	10.610	0.525	11.50	28.750	54.76
3.482	12.124	0.600	13.05	32.625	54.38
3.694	13.646	0.675	14.65	36.625	54.26
3.893	15.155	0.750	16.15	40.375	53.80
4.083	16.671	0.825	17.90	44.750	54.24

Plot of the voltage signal as a function of the power supplied (Fig. 6) showed that there is a linear relationship between them. The slope of the regression line represents the sensitivity of the calorimeter. From the regression line the slope was found out to be 54.12 μV mW^{-1}. The index of correlation (R^2) of the regression line was also found out to be 1.0, showing that there

is a perfect correlation between the power supplied and the voltage signal generated.

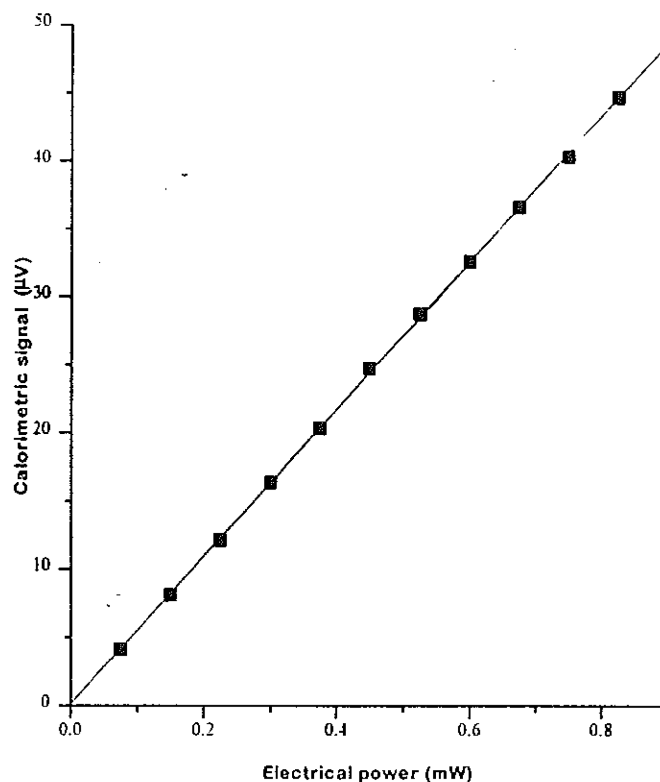


Fig. 6. Plot of the calorimetric voltage signal generated as a function of the power supplied for the electrical calibration of the flow microcalorimeter to determine its sensitivity (S). Equation of the regression line is $y = 0.100 + 54.12 * x$, *and* $R^2 = 1.00$

4.4.2 Effect of inoculum density on the P-t curve

Before investigating the action of metabolic inhibitors on metabolism and hence the P-t curve it was important to establish the P-t curve without inhibitors and study its relation with other metabolic events. Relationship between inoculum density and shape and position of the P-t curve was investigated. In all observations, the P-t curve has two peaks, a smaller peak which appeared earlier and the main peak which appeared 2-3 hours later (Fig. 7).

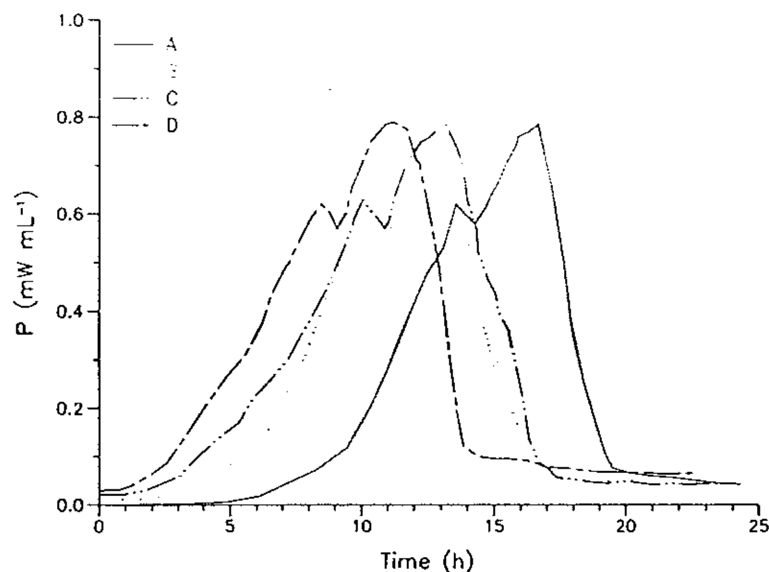


Fig. 7. Heat production rate, P, as a function of incubation time for the flow microcalorimetric growth of *M. luteus* at initial cell densities (cells mL^{-1}) of 7.8 x 10^5 (A); 3.5 x 10^6 (B); 1.4 x 10^7 (C); and 2.4 10^7 (D).

The shape of the P-t curve was the same irrespective of the inoculum density used. With decreasing inoculum density, the P-t curve was shifted to the right more and more with a long calorimetric lag phase at the left.

The power output at the end of the P-t curve didn't return to the baseline, but remained at about 0.04 mW mL^{-1} above the baseline irrespective of the inoculum density.

In addition to its effect on the shape and position, effect of the inoculum density on the P-t curve was also investigated using total heat production during the growth period and cell density at the main peak (Table 2). Each sample was done in triplicate and the mean ± S.D. values were taken.

Table 2. Effect of inoculum density on Q_t (J mL^{-1}) during the growth period and cell density at the main peak (cells mL^{-1}) of the P-t curve of *M. luteus* growing in a flow microcalorimetric system.

Cell density of the inoculum (x10^7 cells mL^{-1})	Q_t ± S.D. (J mL^{-1})	Cell density at the main peak ± S.D. (x10^8 cells mL^{-1})
0.078	18.4 ± 0.45	6.71 ± 0.25
0.086	17.1 ± 0.43	6.75 ± 0.25
0.140	17.8 ± 0.50	6.44 ± 0.26
0.340	17.6 ± 0.48	6.86 ± 0.14
0.920	17.9 ± 0.46	6.35 ± 0.21
1.410	17.4 ± 0.49	6.74 ± 0.17
2.420	18.0 ± 0.47	6.65 ± 0.30
Mean ± S.D.	**17.7 ± 0.39**	**6.64 ± 0.18**

Total heat production during the growth period showed an average value of 17.7 ± 0.39 J mL^{-1} and the average cell number mL^{-1} at the main peak was 6.64 ± 0.18 (x 10^8). Analysis of variance (ANOVA) test was done on both the total heat produced during the growth period and the cell density achieved at the main peak for the different inoculum densities. The calculated F_{value} for the former was found out to be 2.50 and for the latter 3.45. At a level of significance of α = 0.01 and degrees of freedom of k-1 = 6 and k(n-1) = 14, where k = 7 treatments and n = 3 samples per treatment, the tabulated F_{value}, $F_{(0.01)}$ = 4.46. Since $F_{(0.01)}$ is greater than the calculated F_{values} for the treatments the null hypothesis is accepted, i.e., the variations in the average values of Q_t and cell density at the main peak are not introduced due to variations in the inoculum density. Therefore variation in the inoculum size, at the lower inoculum densities used, does not affect the P-t curve except the shift in its position due to length of the calorimetric lag phase.

4.4.3 Relationship between the P-t curve and other features of the culture

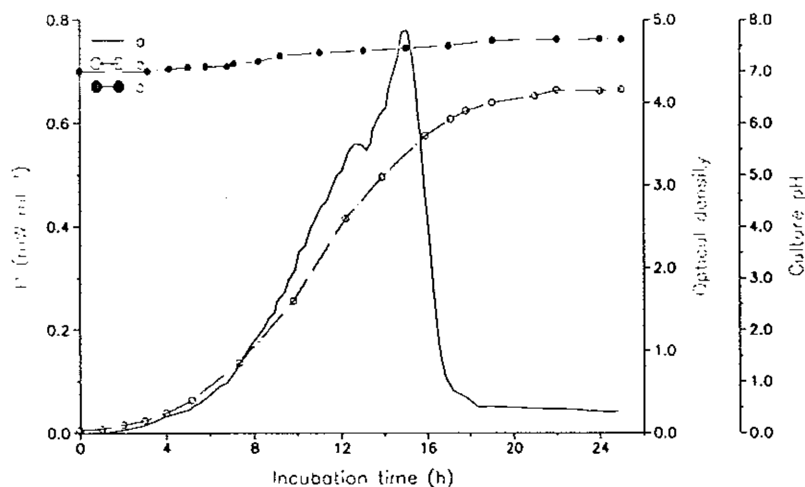
A P-t curve was established by taking an initial cell inoculum of 1.5 x 10^6 cells mL^{-1}.

Samples were taken at different points along the P-t curve to measure, pH, OD and oxygen consumption rate by the batch oxygen electrode.

It was found out (Fig. 8A) that at the beginning of the growth period both the heat production rate and OD were slightly detectable. Both entered the exponential phase after some time was elapsed in the lag phase. Extending the exponential phase of growth to the x-axis and drawing a straight horizontal line along the lag phase parallel to the x-axis gave the length of the lag phase, the x-axis value at which the two lines intersect. Based on this, the lag phase of the P-t curve lasted longer, 5.5 hours, than that of OD, 3.5 hours. After the peak of the P-t curve, as the heat production rate dropped drastically, the slope of the OD curve decreased gradually and entered the stationary phase. The P-t curve remained at a minimum value and the OD curve remained at a maximum value for some time.

During growth in the fermenter the pH of the culture didn't change much (Fig. 8A). A slight drift in pH from 7.0 to 7.6 was observed in 24 hours of growth. The drift in pH was observed in the exponential phase of growth where the metabolic rate of each cell was at its maximum. The more or less constant pH value shows that culture pH was not a factor responsible for the reduction of metabolic rate after the main peak.

A)



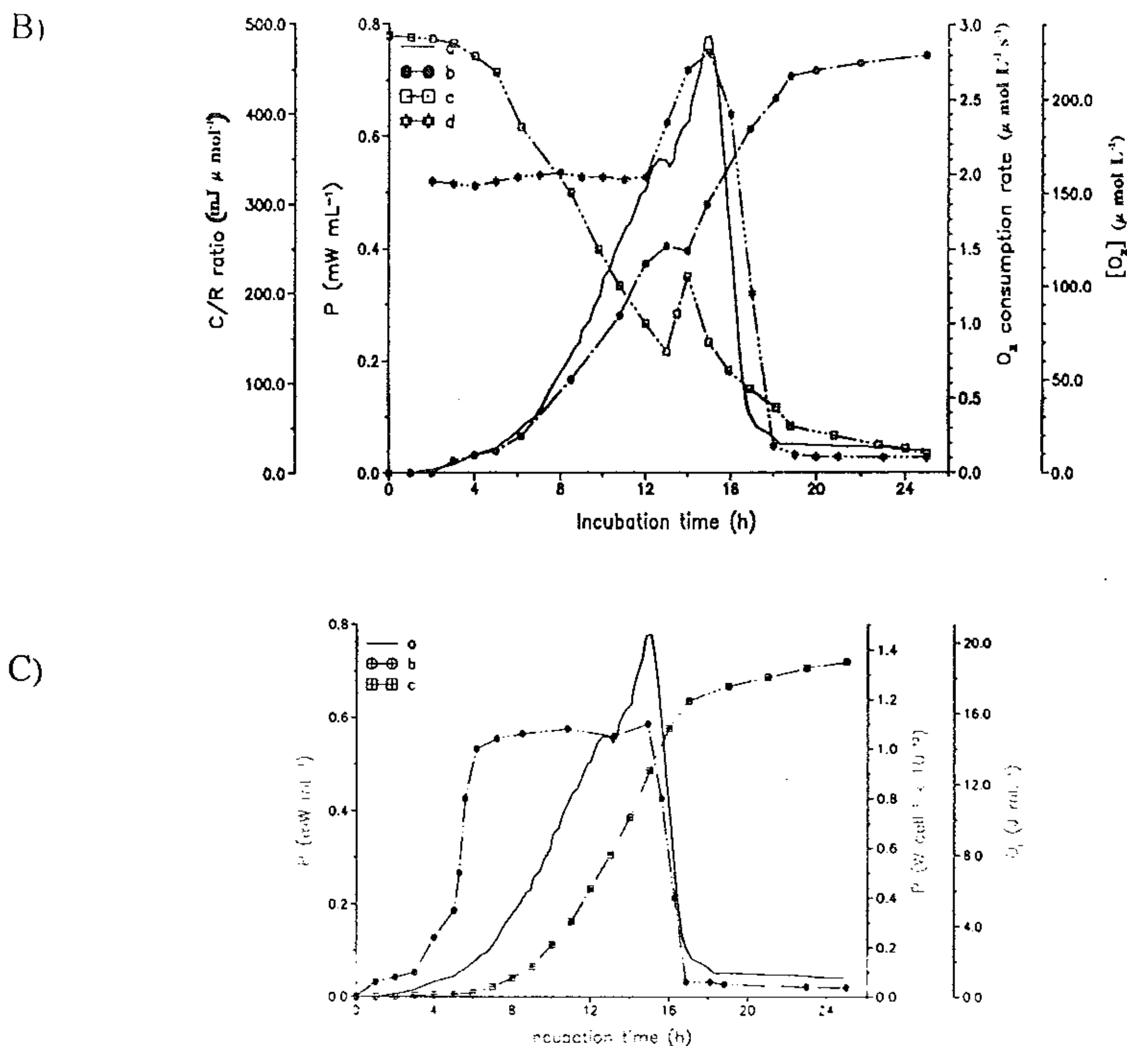


Fig. 8. Flow microcalorimetric growth of *M. luteus* and subsequent changes in features of the culture. (A): heat production rate, P, mW mL^{-1} (a); optical density at 646 nm (b); and pH of the growth medium (c) as a function of time. (B): heat production rate, P, (mW mL^{-1}) (a); oxygen consumption rate (μ mol L^{-1}s^{-1}) (b); oxygen concentration (μ mol L^{-1}) in the flow line (c); and C/R ratio (mJ μmol^{-1}) (d) as a function of time. (C): heat production rate, P, (mW mL^{-1}) (a); heat production rate per cell (W cell^{-1} x 10^{-12}) (b); and total heat production, Q_t, (J mL^{-1}) (c) as a function of time.

The concentration of oxygen in the flow line before inoculation of cells was 230 μ mol L^{-1} (Fig. 8B), near to the air saturation of the liquid medium which was 234 μ mol L^{-1} at 30 °C. This showed that the culture was highly aerated. In the exponential phase of growth, the

concentration of oxygen dropped in the flow line at a high rate up to the first peak of the P-t curve. Immediately after the first peak where the heat production rate dropped by 0.03 mW mL^{-1}, the oxygen concentration in the flow line was raised by 35 μ mol L^{-1} and the oxygen consumption rate dropped by 0.15 μ mol $L^{-1}s^{-1}$. After the trough in the P-t curve, rate of heat production and oxygen consumption increased again and the oxygen concentration started to drop again till it was lowered to 10 μ mol L^{-1} in 24 hours of calorimetric growth. The calorimetric/ respirometric (C/R) ratio obtained by dividing the heat production rate (mW mL^{-1}) by the oxygen consumption rate (μ mol $L^{-1}s^{-1}$ = 1/1000 μ mol $mL^{-1}s^{-1}$) remained constant at *ca* 330 mJ μ mol^{-1} in the lag and exponential phase before the first peak. Immediately after the first peak the C/R ratio increased to 390 mJ μ mol^{-1}. This ratio increased to 470 mJ μ mol^{-1} at the main peak. Then the C/R ratio declined with the falling of the P-t curve and remained at a low value, 18 mJ μ mol^{-1}, after the P-t curve has dropped to about 0.04 mW mL^{-1} and the oxygen consumption rate became more or less constant. The constant oxygen consumption rate after the main peak, determined by the batch oxygen electrode, may be an indication that cells have entered the stationary phase of growth.

The heat production rate by each cell showed variation at different points along the P-t curve (Fig. 8C). In the lag phase of the P-t curve the heat production rate per cell remained at a low value. It was observed that in the transition from the lag to the exponential phase there is an abrupt increase in heat production rate per cell to achieve a maximum value of 1.05×10^{-12} W $cell^{-1}$. The rate was maintained at this value through out the exponential phase of growth; it then started to drop down immediately after the peak of the P-t curve and returned to a minimum value. The minimum value remained at *ca* 0.04×10^{-12} W $cell^{-1}$ at least for six hours.

The total heat production (Fig. 8C) showed the same pattern as the OD (Fig. 8A) except that the latter achieved a horizontal curve after 22 hours. In the former case, however, the curve showed an increase, though at a very low rate, after the main peak of the P-t curve. This increase continues as long as there is heat production though the rate is very low.

4.4.4 Effect of EEP on the P-t curve.

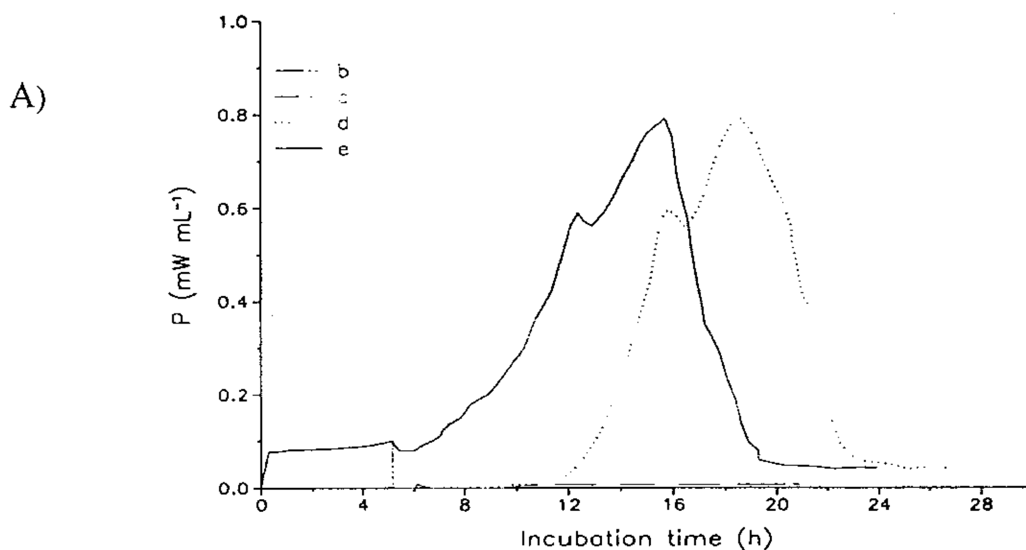
4.4.4.1 Effect at different phases of the P-t curve

The effect of EEP at different phases of the P-t curve was investigated by using

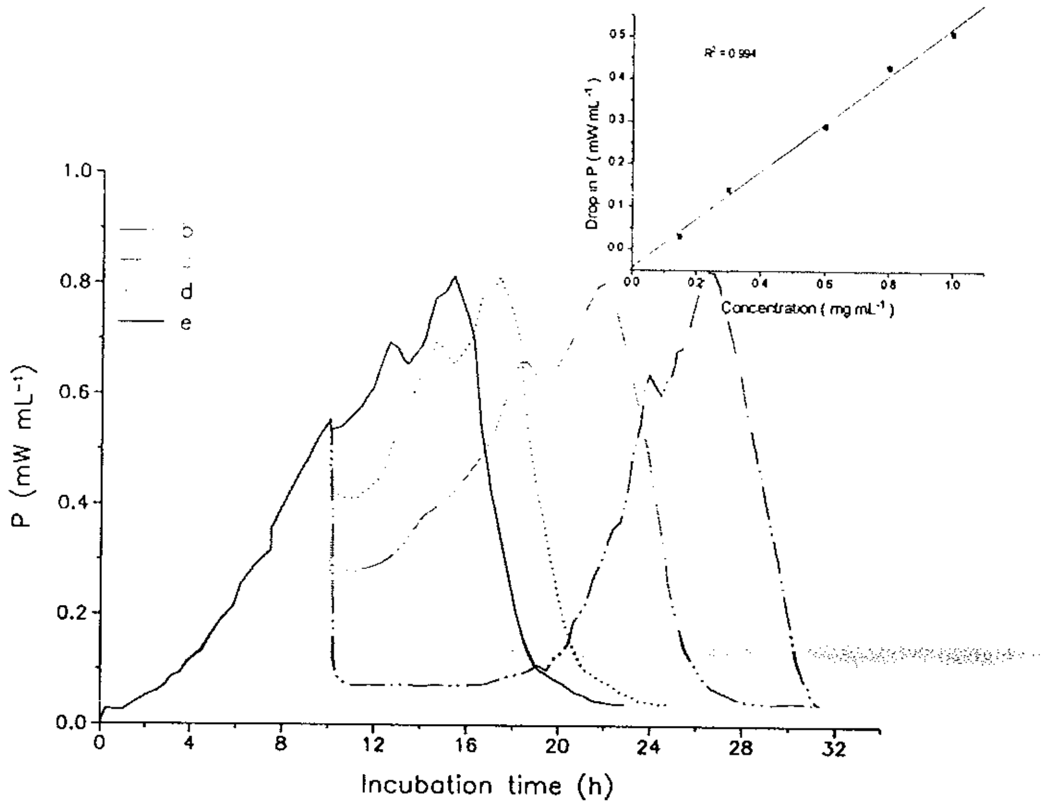
varying concentrations of tincture (Fig. 9). Addition of higher concentration of tincture at the late lag phase of growth (Fig. 9A) resulted in a drop of the P-t curve to the baseline and growth was not resumed with in at least 15 hours. For lower concentrations, 0.3 and 0.15 mg mL^{-1}, growth was resumed after 5.7 and 1.0 hours, respectively. For higher concentrations (1.0, 0.8, and 0.6 mg mL^{-1}), comparison of their effects was not possible since all of them resulted in a drop of the P-t curve to the base line.

Effects of the different concentrations of tincture were also observed by adding them at the mid exponential phase of growth (Fig. 9B). Tincture was added at the mid exponential phase, which corresponded to a power output of 0.54 mW mL^{-1}. At this phase of the P-t curve effects of the different concentrations of tincture were seen clearly. Hence this phase was used for the comparison of effects of different propolis on the P-t curve (see section 4.4.4.2).

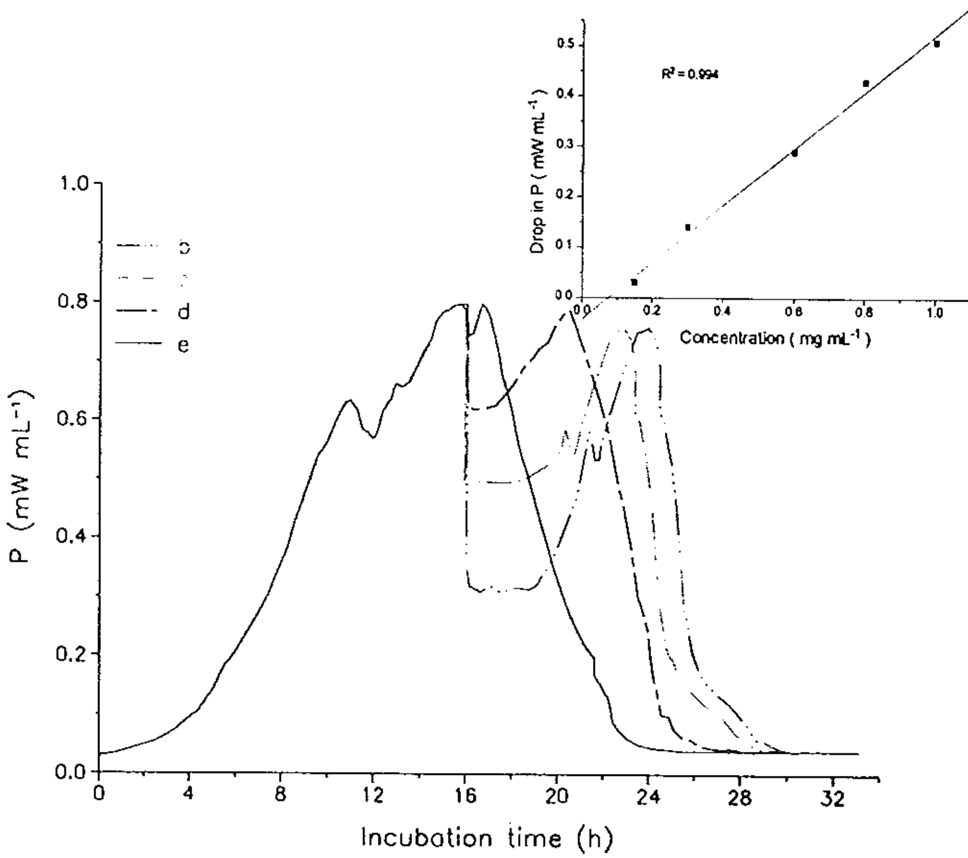
A)



B)



C)



D)

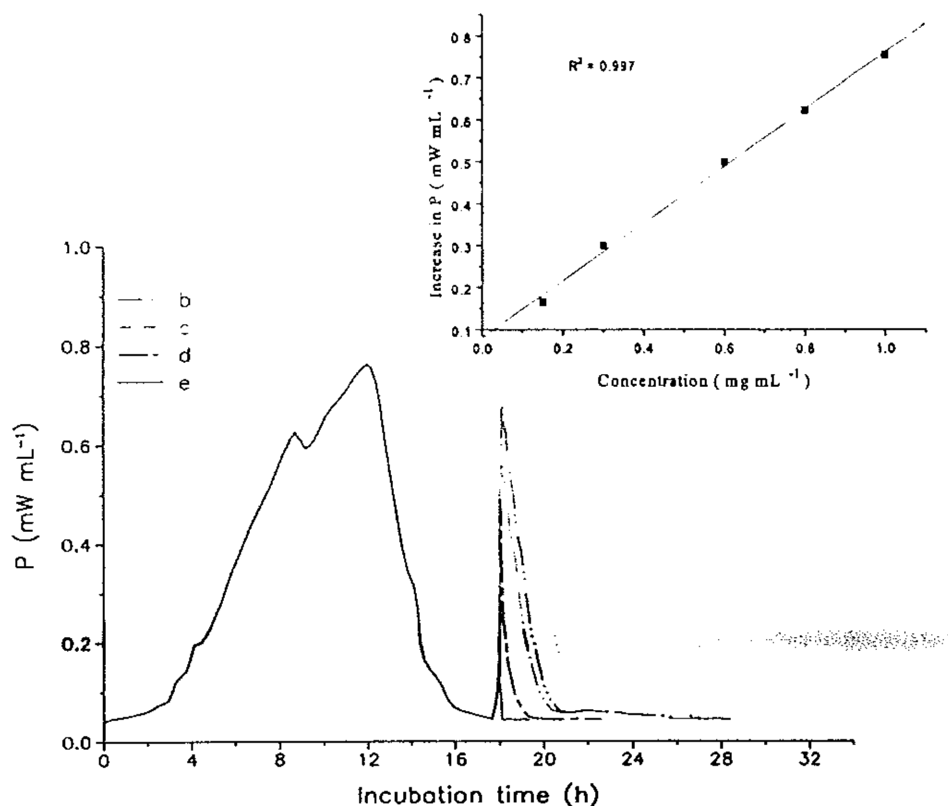


Fig. 9. Effect of propolis tincture at different phases of the P-t curve of *M. luteus* in a flow microcalorimeter. Tincture was added at the lag phase (A); mid exponential phase (B); immediately after the main peak (C); and after the fall of the P-t curve to a minimum value (D). The insets are dose-response curves interms of the fall in power output (B and C) and increase in power output (D) due to addition of various concentrations of tincture. The concentration (mg mL^{-1}) of tincture added at each phase of the P-t curve included 1.0 (a); 0.8 (b); 0.6 (c); 0.3 (d) and 0.15 (e).

Addition of tincture immediately after the main peak (Fig. 9C) resulted in the same effect as at the mid exponential phase of growth. For a given concentration, the drop in power output after treatment with tincture was the same irrespective of the phase at which propolis was added (mid exponential phase or after the peak). However, the time needed for the resumption of growth showed variation with the phase at which tincture was added. This was the case especially at higher concentrations where the P-t curve dropped nearby to the base line and hence the time needed for the resumption of growth was longer when tincture was added at the exponential phase than after the peak where the curve remained at a higher value than in the former case. When the concentration of tincture used resulted in a drop of the P-t curve below the level of the first peak and if growth resumed (Fig. 9C [b and c]) the curve showed

the appearance of the first peak followed by the main peak as in the control curve. However, in cases where the curve didn't drop below the level of the first peak, no peak equivalent to the first appeared (Fig. 9C [d and e]).

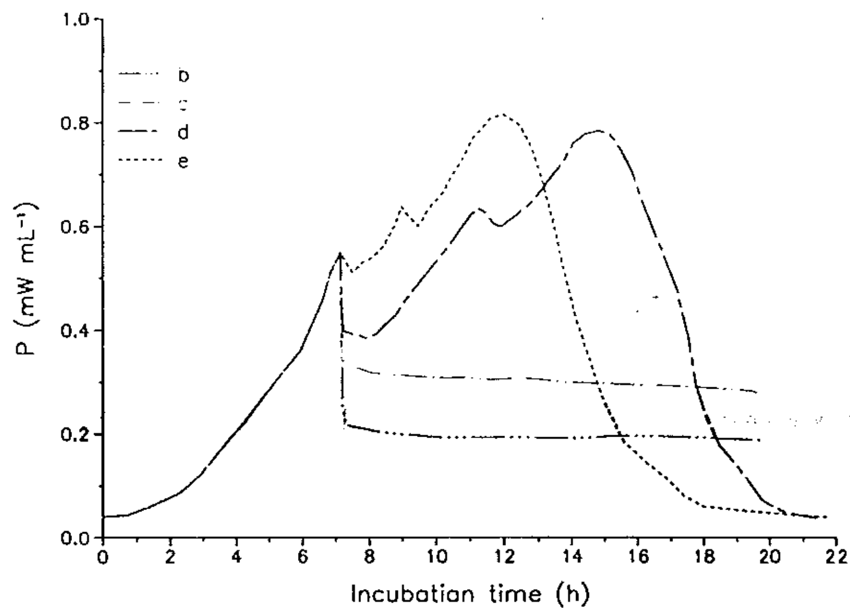
Addition of tincture after the fall of the curve to a minimum value resulted in an increase in power output very rapidly (Fig. 9D). With an increasing concentration of tincture an increase in power output was observed. The curve returned to a minimum value sooner with rate of drop of the curve comparable to that of the drop of the curve from the main peak in the control curve. Hence assay of antibacterial activity can also be done at this phase of the P-t curve for moderate or lower concentrations because higher concentrations may give complicated results due to killing of almost all cells. This is because the P-t curve may not show response due to killing of all cells and may be confused with those that do not have antibacterial effects or very low concentrations of antibacterial agents below their MIC value. But very high concentrations were not included in the present work.

The insets in Fig. 9B, C, and D are dose-response curves of tincture added at the corresponding phases. It was not possible to construct a dose-response curve for tincture added at the late lag phase due to drop of the P-t curve to the baseline in almost all cases except at very low concentrations.

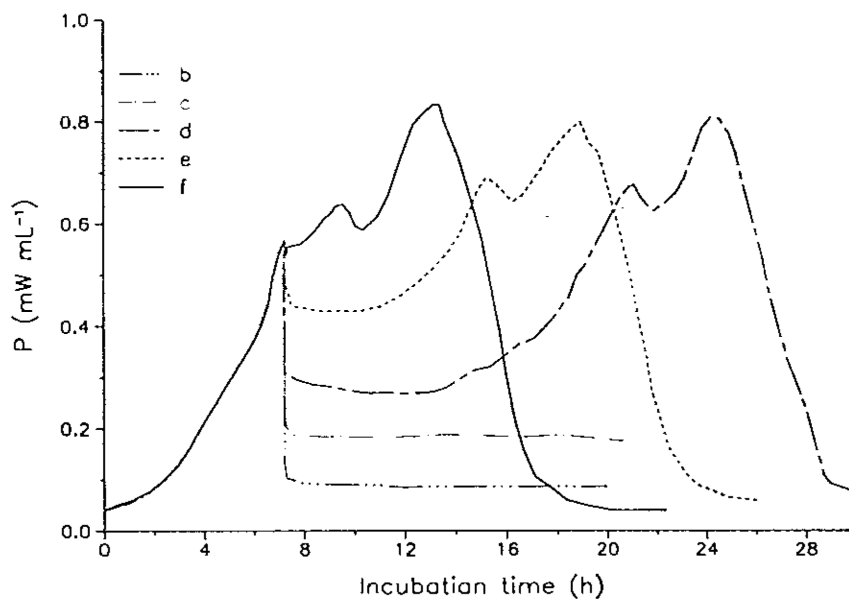
4.4.4.2 Comparison of effect of different EEP at the mid exponential phase of the P-t curve

The addition of EEP to growing cells of *M.luteus* at the mid exponential phase resulted in P-t curves which differed from the control curve in a manner which was dependent up on the nature and concentration of EEP applied (Fig. 10 A-D). As soon as EEP was added to the culture some cells were killed and consequently heat production rate declined.

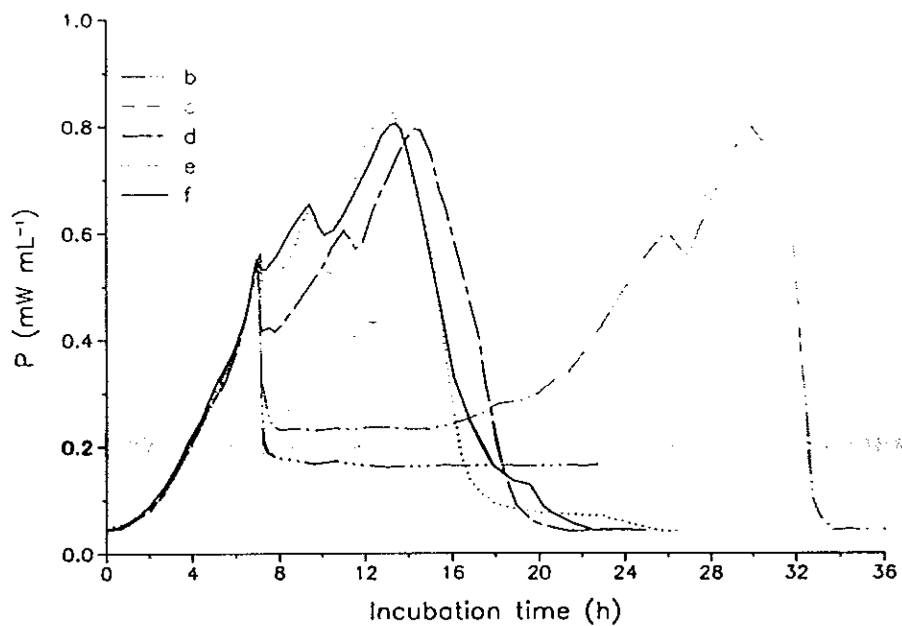
A)



B)



C)



D)

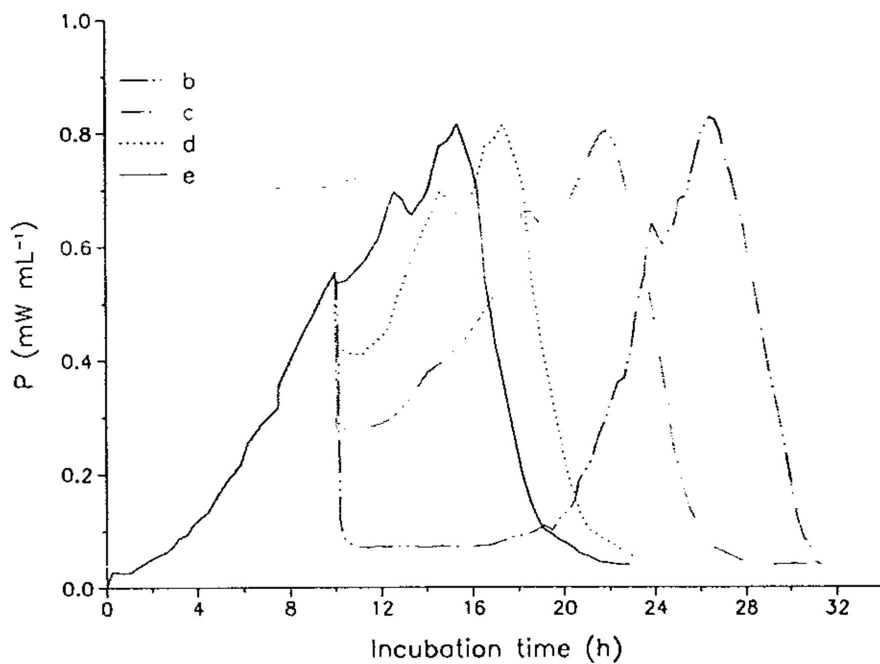


Fig. 10. Effect of EEP of prop1 (A); prop2 (B); prop3 (C); and tincture (D) on the P-t curve of flow microcalorimetric growth of *M. luteus*. The concentrations (mg mL^{-1}) used included 1.0 (a); 0.8 (b); 0.6 (c); 0.3 (d); 0.15 (e); and 0.072 (f).

At a higher concentration of EEP (1.0 mg mL^{-1}) the heat production rate dropped near to the baseline and remained there for at least 15 hours for all propolis samples used. With decreasing concentration of EEP the drop in heat production rate decreased and at lower concentrations cellular growth was resumed.

In case of prop1 cell growth was not resumed at least for 15 hours at concentrations ≥ 0.6 mg mL^{-1} (Fig. 10A [a.b and c]). At lower concentrations, however, though the P-t curve showed a drop as EEP was added cell growth and metabolism was resumed later on (Fig. 10A [d and e]). The time required for the resumption of growth and increase in the P-t curve showed dependence on the concentration of EEP added. At lower concentrations of EEP cell growth and metabolism was resumed soon. At these lower concentrations the P-t curves after the resumption of growth were similar to the control in that they showed a small peak before the main peak and both of these peaks appeared at power values similar to the control. The small peak appeared at 0.6 ± 0.04 mW mL^{-1} and the main peak at 0.8 ± 0.035 mW mL^{-1}. At the end of growth the P-t curve returned to a lower value of *ca* 0.04 mW mL^{-1} as in the case of the control. Effect of EEP of prop1 on metabolism was observed up to a concentration of 0.15 mg mL^{-1}; concentrations less than this didn't show any effect on the P-t curve. Hence the minimal inhibitory concentration (MIC) of prop1 is 0.15 mg mL^{-1}.

Prop2 showed the same pattern as prop1 at concentrations ≥ 0.6 mg mL^{-1} and ≤ 0.3 mg mL^{-1} (Fig. 10B). However, in this case the time needed for the resumption of growth was longer as compared with that of prop1. Measuring the length of the trough between the minimum point after addition of EEP and the point at which increase in the P-t curve was detected gave the time needed for the resumption of growth. Based on this the time needed for growth to be resumed after treatment with concentrations of 0.3 and 0.15 mg mL^{-1} EEP were 0.86 and 0.29 hours, respectively, for prop1; and 6.25 and 2.75 hours, respectively, for prop2; and 0.5 hour for 0.072 mg mL^{-1} prop2.

The P-t curve for prop2 after the resumption of growth showed the same pattern with the control and prop1. The MIC value for prop2 was found out to be 0.072 mg mL^{-1}, which is half of that of prop1.

For prop3 growth was prevented and the P-t curve remained at a lower position for concentrations of 1.0 and 0.8 mg mL^{-1} for at least 15 hours. At concentrations ≤ 0.6 mg mL^{-1} growth was resumed (Fig. 10C). The time needed for the resumption of growth was 7.8, 0.8, 0.28 and 0.15 hours after treatments with concentrations of 0.6, 0.3, 0.15 and 0.072 mg mL^{-1},

respectively. The pattern of the curve after the resumption of growth was the same as for the control and others seen previously. The MIC was found out to be 0.072 mg mL^{-1}.

Tincture added at the exponential phase showed that only for 1.0 mg mL^{-1} growth was not resumed and hence the P-t curve remained at a lower position for at least 15 hours (Fig. 10D). At concentrations $\leq$ 0.8 mg mL^{-1} growth was resumed with in 15 hours or less and hence an increase in the P-t curve was observed and a pattern similar to the control was achieved. The time needed for the resumption of growth after treatment with concentrations of 0.8, 0.6, 0.3 and 0.15 mg mL^{-1} corresponds to 8.25, 2.8, 1.7 and 0.3 hours, respectively. The MIC was found out to be 0.15 mg mL^{-1}.

In general, for all propolis samples, with decreasing concentration of EEP the drop in the P-t curve decreased. A dose- response curve was established based on the drop in the P-t curve as a function of concentration of EEP (Fig. 11).

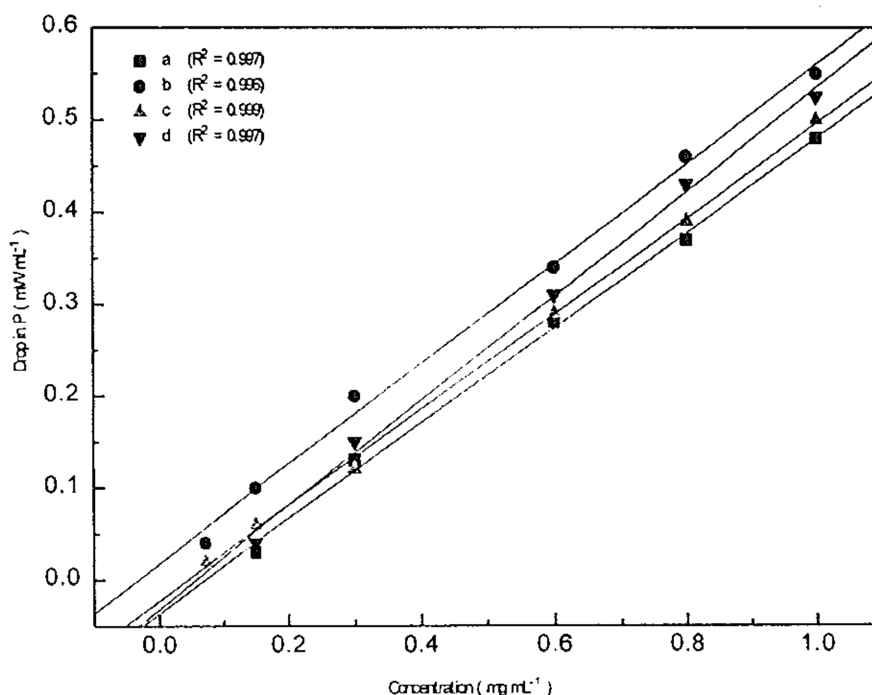


Fig. 11. Dose-response curve for EEP of prop1 (a); prop2 (b); prop3 (c); and tincture (d) added at the mid exponential phase of growth of *M. luteus* in a flow microcalorimeter. The response was measured by the drop in power output from the point of addition of EEP to the minimum point achieved in the curve due to EEP addition.

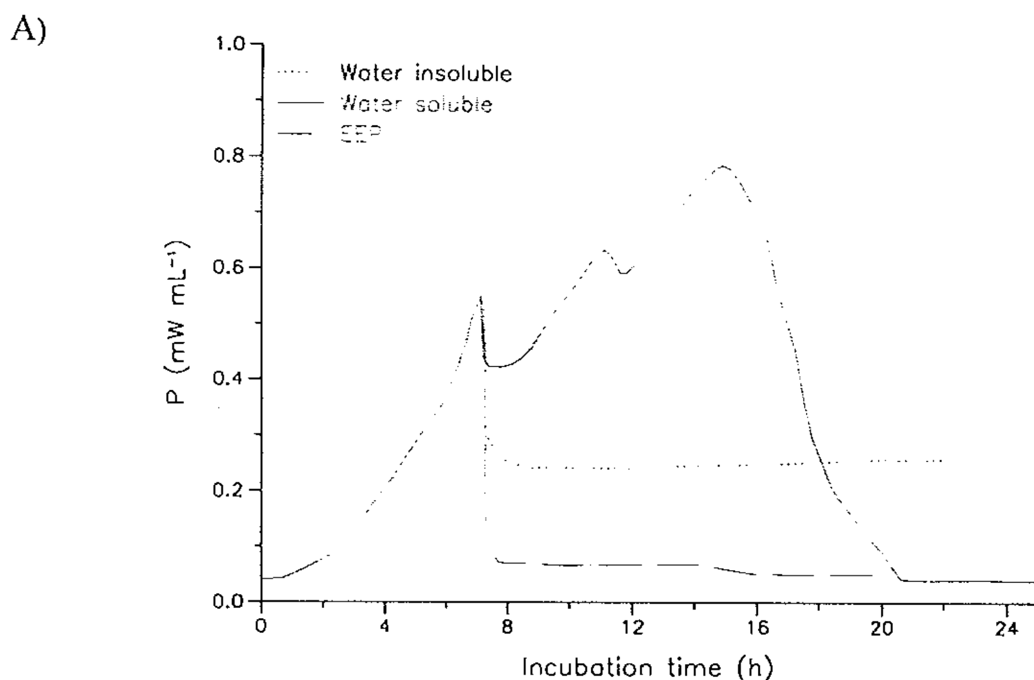
It can be seen from Fig. 11 that all EEP samples used have the same pattern of dose-response curves. Prop2 showed a slightly higher effect than the others and as a result the curve lied above the other curves. The strength of antibacterial activity can be sequenced as prop2 > tincture > prop3 > prop1 based on the drop of the curve from the point of addition of EEP to a minimum value. Though a difference was observed on their effect on the P-t curve, as sequenced above, it was a slight difference.

The 60% ethanol solution used as a control didn't show any effect on the P-t curve indicating that it didn't add effect to the antibacterial activity of propolis.

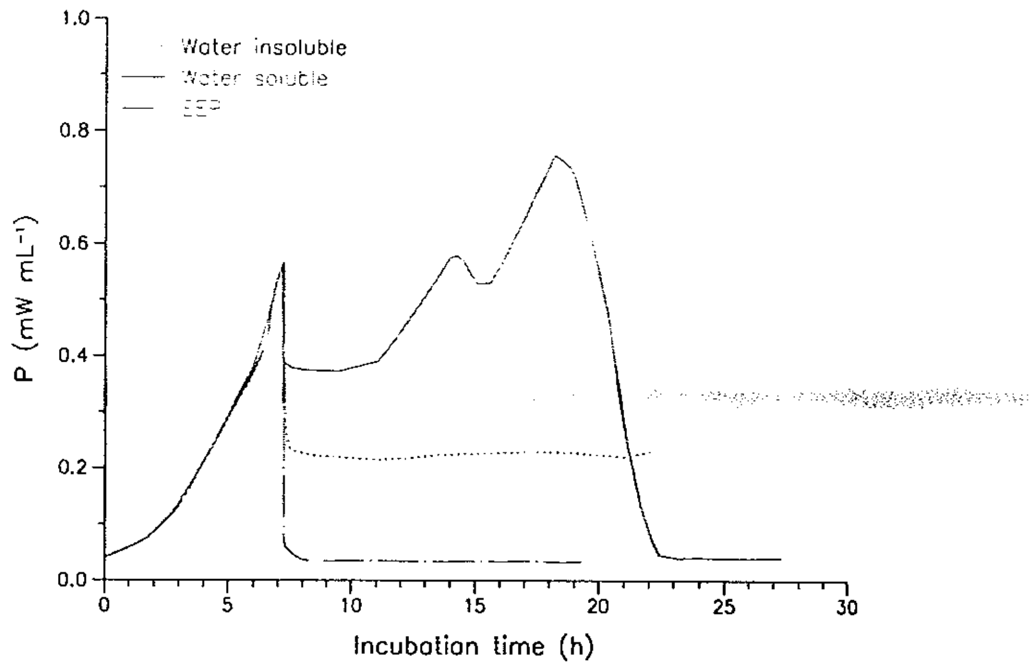
4.4.5 Comparison of the effect of water soluble and insoluble components of EEP

For the sake of comparison of their effect on the P-t curve 1 mg mL^{-1} of water soluble and insoluble components as well as the unprecipitated EEP were incorporated at the mid exponential phase of growth (Fig. 12).

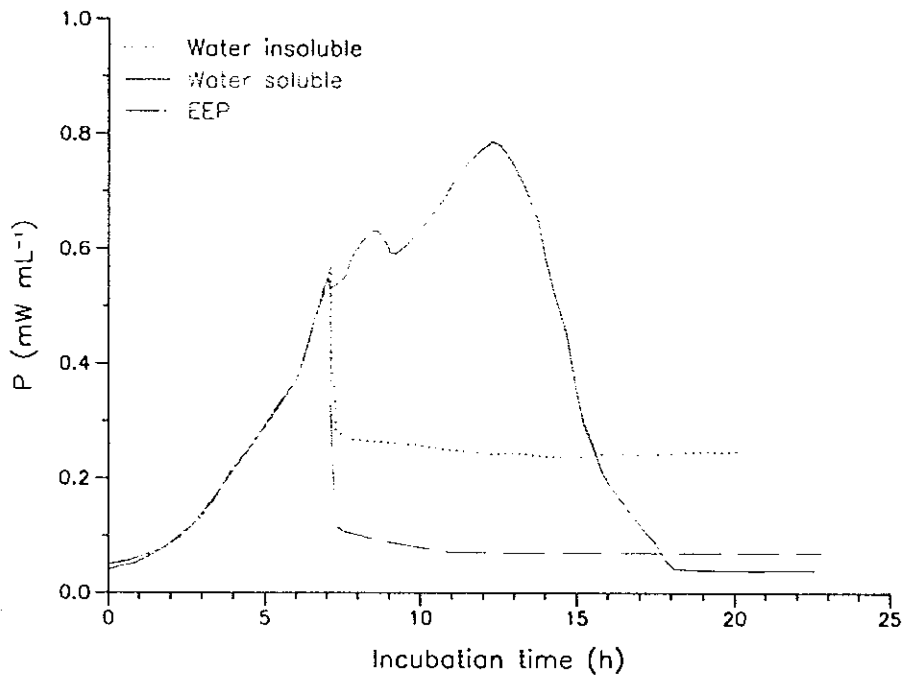
A)



B)



C)



D)

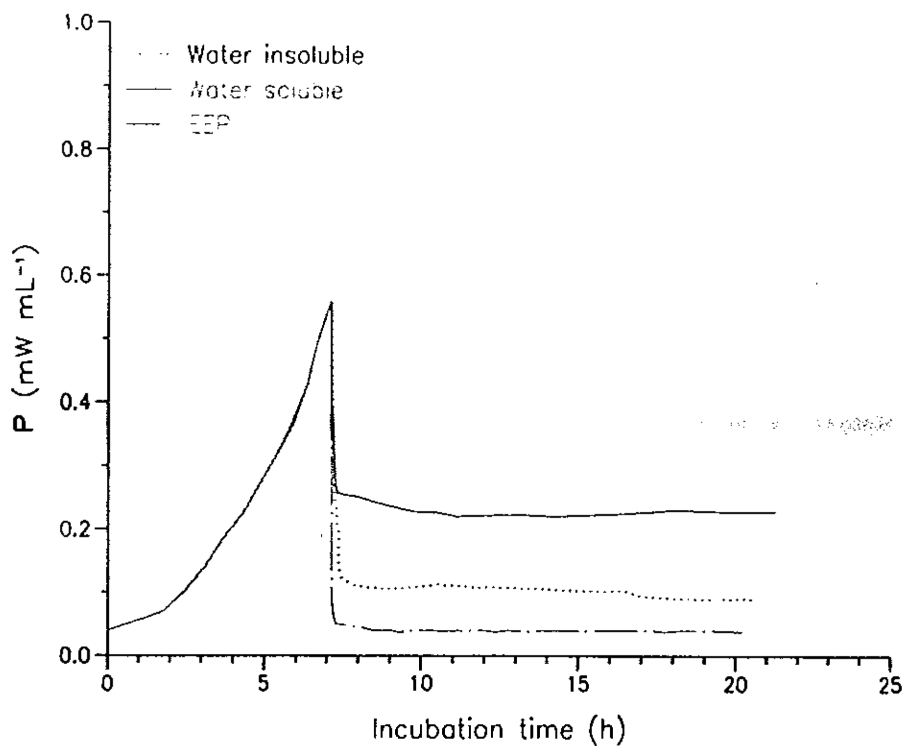


Fig. 12. Effect of water soluble component, water insoluble component, and unprecipitated EEP of prop1 (A); prop2 (B); prop3 (C) and tincture (D) on the P-t curve of *M. luteus* grown in a flow microcalorimeter. Samples were injected at the mid exponential phase of growth.

In all cases, the unprecipitated EEP showed a higher antibacterial activity as compared to the water soluble and insoluble components. The drop in power output achieved due to addition unprecipitated EEP was even higher than that of the sum of the two components except for tincture where the sum was slightly higher than that for unprecipitated EEP (Table 3).

Table 3. Drop in power output (mW mL^{-1}) achieved due to incorporation of 1 mg mL^{-1} different propolis preparations at the mid exponential phase of growth of *M. luteus* in a flow microcalorimeter.

Type of propolis	EEP	H_2O insoluble component	H_2O soluble component
Prop1	0.48	0.33	0.11
Prop2	0.53	0.33	0.14
Prop3	0.49	0.30	0.02
Tincture	**0.51**	**0.45**	**0.32**

Both the water soluble and insoluble components of tincture showed a higher activity than the corresponding components in other propolis samples. Growth was resumed after treatment with the water soluble components of EEP except that of tincture which didn't show any growth with in 14 hours after treatment. The water soluble component of prop3 has very low antibacterial activity as compared to the same component from other propolis samples.

MIC values for the water soluble and insoluble components were determined and found out to be 0.6 and 0.15 for prop1 and also for prop2; 1.0 and 0.15 for prop3; 0.3 and 0.15 mg mL^{-1} for tincture, respectively. The MIC values of unprecipitated EEP, determined in the last section, were the same as that of the water insoluble components in the case of prop1 and tincture; but half in the case of prop2 and prop3. MIC values for the water soluble components were higher than their EEP complements in all cases.

4.4.6 <u>Effect of ampicillin</u>

Sodium salt of ampicillin (amino benzyl penicillin sodium salt), a semisynthetic antibiotic, active against both Gram positive and Gram negative bacteria was injected at the mid exponential phase to compare its effect with that of propolis.

The mechanism of action of ampicillin (Fig. 13) was found out to be different from that of EEP (see section 4.4.4 and 4.4.5). In case of all EEP used the P-t curve dropped to a minimum value immediately after the addition of propolis. However, in case of ampicillin no detectable effect was observed on the P-t curve for 2-3 hours after treatment. After this time

the rate of increase in power output slowed down and entered at a plateau phase where no further increase in power output was observed. After a stay for some time, in the plateau phase, the power output started to fall down. The length of the plateau phase is inversely proportional to the concentration of ampicillin added (see the inset in Fig. 13). At a concentration of 0.072 mg mL^{-1} the curve remained at a plateau phase (at 0.68 mW mL^{-1}) till to the end of the experiment (20 hours after treatment).

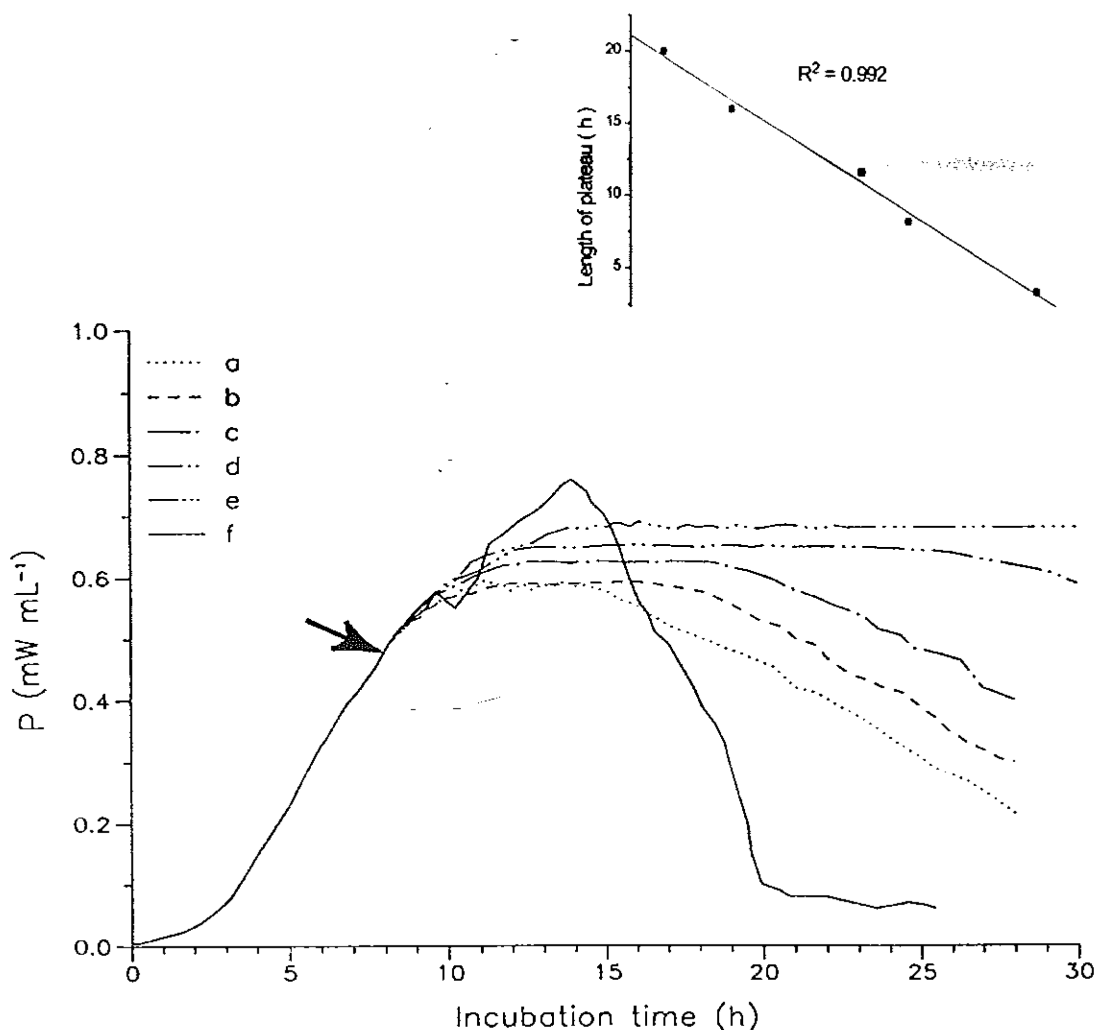


Fig. 13. Effect of ampicillin sodium salt on the P-t curve of *M. luteus* grown in a flow microcalorimeter. The final concentrations (mg mL^{-1}) of the antibiotic in the culture amounted to 4 (a); 1 (b); 0.6 (c); 0.15 (d); 0.072 (e); and 0.005 (f). The arrow shows point of addition of ampicillin in the P-t curve. The inset shows the dose-response curve interms of concentration of ampicillin versus length of the plateau phase after the effect was observed and before decline in the P-t curve started.

After treatment with 0.005 mg mL^{-1} the shape of the curve didn't show much difference

from the control except that the first peak was not clearly visible and the main peak was achieved at a value of 0.75 mW mL^{-1} which was less than that for the control (0.8 ± 0.03 mW mL^{-1}). In addition to these the minimum value achieved after the fall of the P-t curve was slightly higher than for the control.

Concentrations less than 0.005 mg mL^{-1} didn't show any effect on the P-t curve. Hence the MIC value is 0.005 mg mL^{-1}. This MIC value is very low as compared to the MIC values for all propolis samples used showing that ampicillin is effective even at lower concentrations.

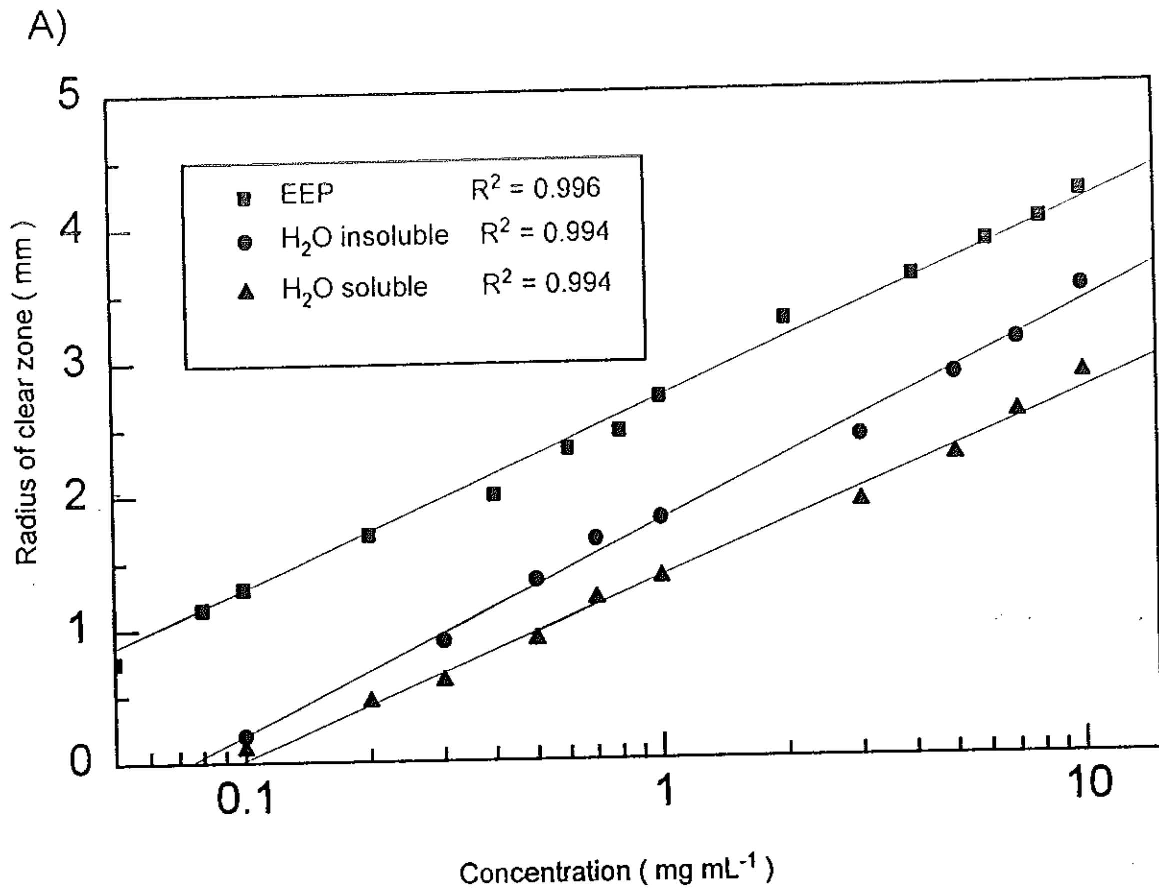
4.4.7 Microscopic examination of cell morphology

Microscopic examination of cell morphology was done before and after treatment with EEP, water soluble and insoluble components of EEP and ampicillin by taking a sample at the outlet of the flow line. Before treatment, 3 or more cells sticked together and cells rarely occurred as single cells. After treatment, however, cells were found as single cells and rarely in a group of 2 or more. At higher concentrations, no adherent forms were observed indicating that treatment with the antibacterial agents may affect the cell wall. Treatment with 60% ethanol didn't show any visible change on the appearance of cells.

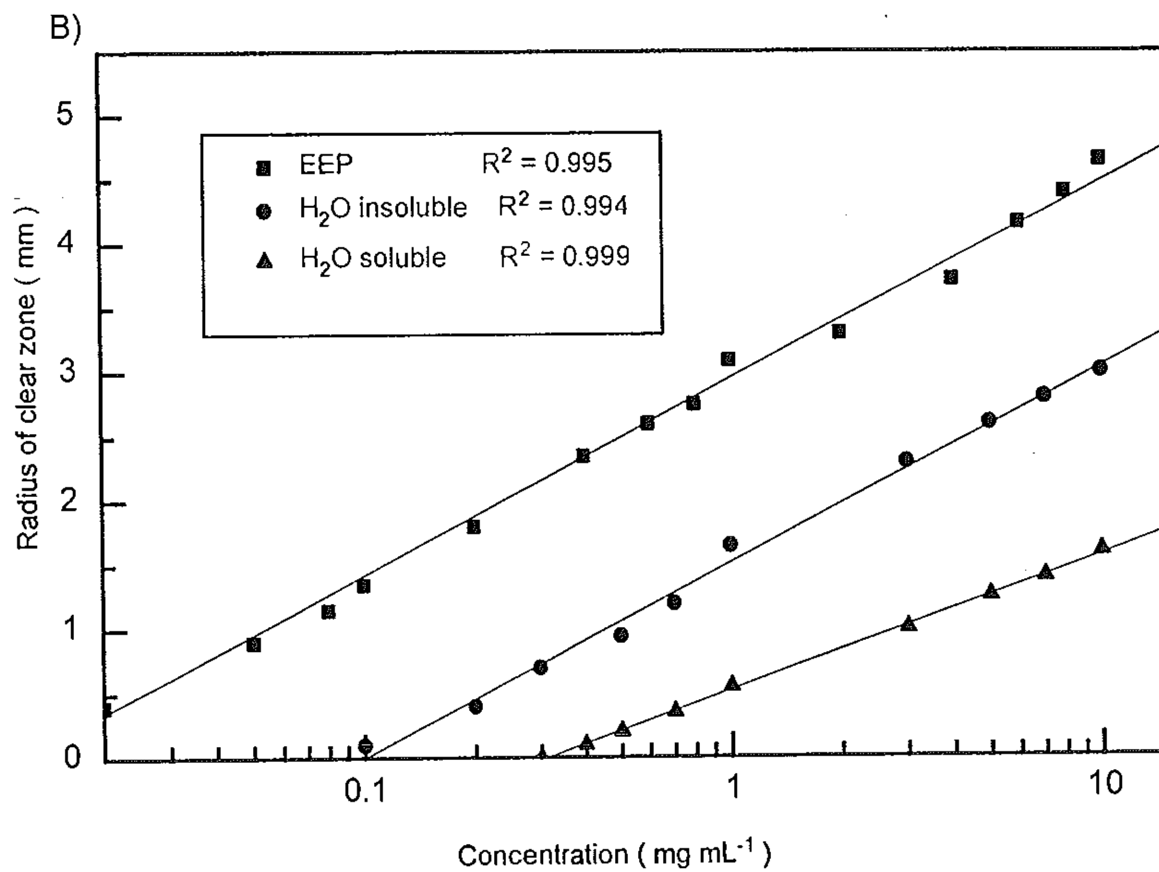
4.5 AGAR WELL DIFFUSION ASSAY

The dose-response curves obtained from plotting the concentration of EEP, water soluble and insoluble components of EEP and ampicillin against the resultant inhibition of bacterial growth are shown in Fig. 14. The R^2 value for each graph is given as an indication of the reproducibility of the experiment. A linear response was obtained in all of the tests except at a higher concentration of prop3 (for EEP and its water insoluble component) where saturation effect was observed at ≥ 5 mg mL^{-1}. The clear zone produced by ampicillin at a given concentration was very large as compared to that by the different propolis samples used. At the end of the incubation period precipitates were observed in the wells for plates incubated with EEP and its water insoluble component. The amount of precipitate increased with increasing concentration of the component used (only observation, data not incorporated). However, no precipitates were observed for the water soluble components of EEP and ampicillin.

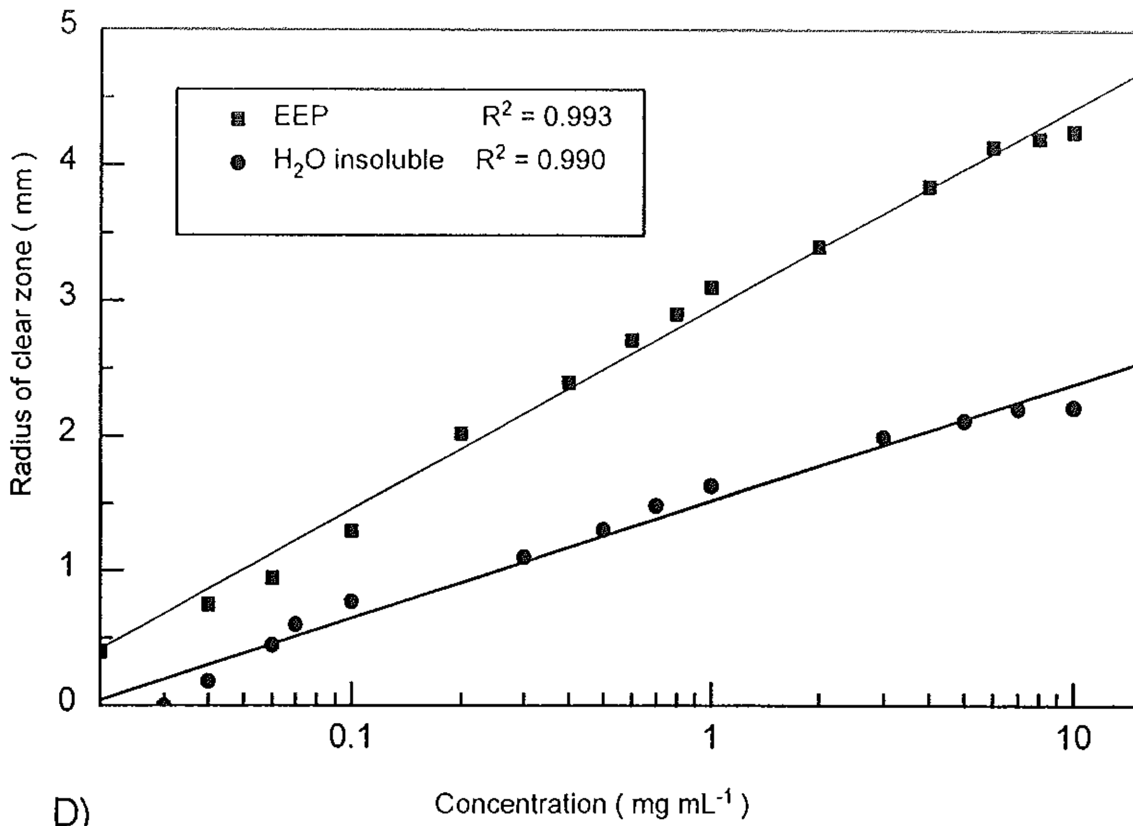
A)



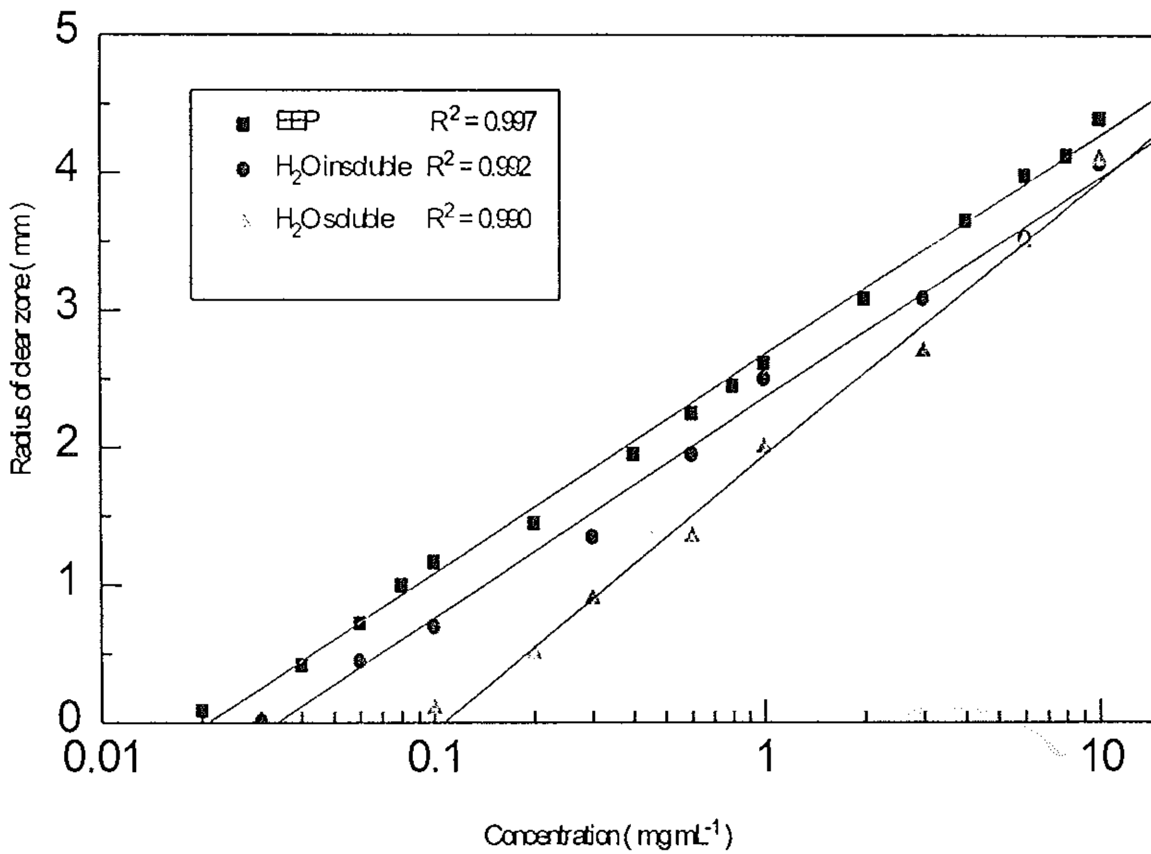
B)



C)



D)



E)

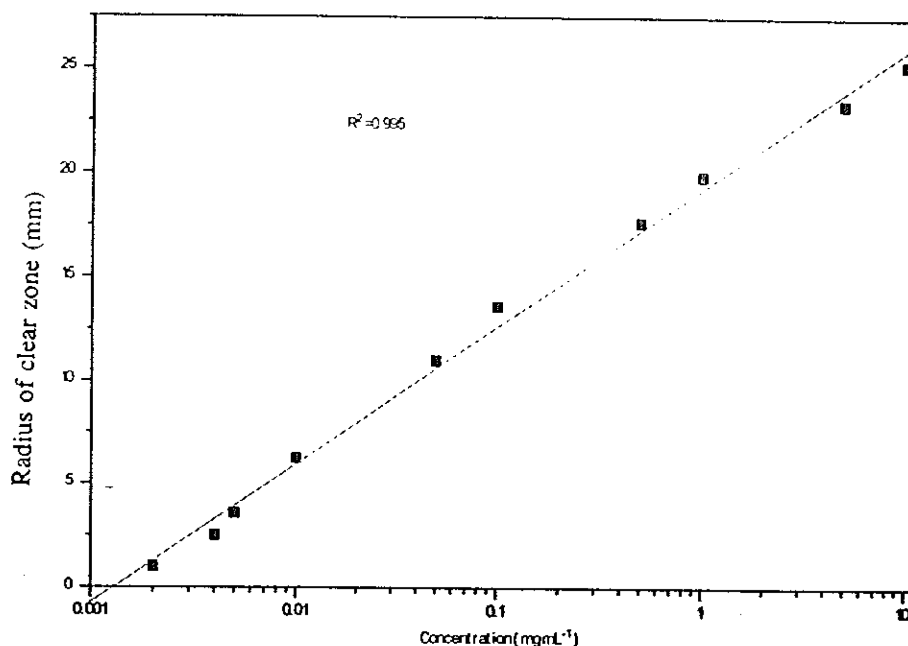


Fig. 14. Dose-response curves for the antibacterial activity of prop1 (A); prop2 (B); prop3 (C); tincture (D); and ampicillin (E) for *M. luteus* using the agar well diffusion assay method. For all propolis samples both EEP and their water soluble and insoluble components were used.

For all propolis samples used EEP has a higher activity than its water soluble and insoluble components in that the dose-response curve for the former lies above the other curves and the MIC for the former is lower in all cases than the other two. In case of prop3 the water soluble component didn't show any activity within the range tested; however, activity was observed at higher concentrations ($\geq$ 20 mg mL^{-1}), data not incorporated. The water insoluble component showed a higher activity than the water soluble component for all propolis samples used except at about 10 mg mL^{-1} of tincture where both showed an almost equal activity.

Variation in activity was observed among the water insoluble components of the different propolis samples. This component from tincture showed a higher activity having an MIC value of 0.03 mg mL^{-1} which is also the same for prop3, where the corresponding values for the others were higher (0.1 mg mL^{-1} for prop1 and prop2). Though no variation was observed on the MIC values between the water insoluble components of prop3 and tincture there was variation in activity with increasing concentration. For example at 10 mg mL^{-1} the

clear zones were 2.2 mm and 4.1 mm for prop3 and tincture, respectively. The water soluble components also showed variation in activity with the maximum activity observed in the component from tincture.

There was no much difference in activity between the different EEP samples used. The MIC values were the same for all, 0.01 mg mL^{-1}, except for tincture which was slightly higher, 0.02 mg mL^{-1}. The maximum activity within the range tested lied between 4.2 mm and 4.6 mm clear zone radius for all EEP used showing no great variation. Therefore, all EEP used didn't show much difference in activity though difference in activity was observed between the water soluble and insoluble components of a given EEP and among the water soluble and insoluble components of different EEP.

5. DISCUSSION

M. luteus was chosen for the present work because it was not reported to be resistant to any sort of antibiotics and hence recommended for use to test antibacterial activity by the German collection for microorganisms and cell cultures (DSM, 1989 Catalogue). In addition to this since the institute where the calorimetric experiments had to be done was not a medical institute, it was not safe to use pathogenic organisms, though the results were to be recommended for use against pathogenic microorganisms.

Cell growth and reproduction are governed by different cellular activities which are affected by external factors such as temperature. With most microorganisms three cardinal temperatures can be identified; the minimum, optimum and maximum. The minimum temperature of growth may depend on solidification of membrane lipids, or on the marked sensitivity of the initiation process in protein synthesis to cooling (Marr and Ingraham, 1962). Slightly above the maximum temperature, in contrast, many enzymes are denatured and the cell dies. Since lower temperatures are not lethal, (refrigeration of microbial cultures stems from this fact), the minimum growth temperature for *M. luteus* was achieved gradually and hence the curve was extended to lower temperature values (Fig. 3). However, since higher temperature values are lethal, the maximum temperature (45 °C) was achieved slightly above the optimum range. Decrease in OD was observed at temperatures above the maximum. This decrease could be due to cell death and lysis at higher temperature values.

Propolis samples from different geographical origins (Ethiopia, Germany and Uraguay) gave a comparable yield of 21% up on extraction with 70% methanol. Though these propolis samples were collected from countries with different climate and vegetation and could differ in their chemical composition, the basic make up of propolis: 55% resin, 30% wax, 10% essential oils and 5% pollen is maintained regardless of the geographical origin of propolis (Ghisalberti *et al.*, 1978). As a result, the percentage of alcohol extractable compounds remains constant. EEP possesses both water soluble and insoluble compounds. The percentage of either of the components of EEP varies as far as the plant source of propolis is not the same. Since all propolis samples used were collected from different geographical localities the variation in the percentage of water soluble and insoluble components in EEP is not surprising.

The presence of water insoluble components in propolis limits its clinical applications (Dimov *et al.*, 1992) especially as a drug to be administered orally. As a solution for this a

water soluble derivative (WSD) of propolis was prepared (Nikolov *et al.*, 1991). However, since results from both calorimetry (Fig. 12 A - D) and agar well diffusion assay (Fig. 14 A - D) showed that the water soluble components were inferior in activity to EEP and water insoluble components, the full potential of propolis is not utilized while using the WSD of propolis as an antibacterial agent.

Inoculation of cell suspension into the fermenter, connected to the flow microcalorimeter, after establishment of a stable baseline showed a lag phase which is represented by the absence of increase in power output through time, at the left base of the P-t curve. Inoculation of a cell suspension of less than 10^6 cells mL^{-1} didn't show any response and the P-t curve lied at the baseline. At cell densities $\geq 3.5 \times 10^6$ cells mL^{-1} as an inoculum, however, the recorder showed deflection from the baseline indicating that there is heat production (Fig. 7). The deflection from the baseline increased with increasing inoculum density. These results show that the cells were metabolizing as they were transferred to the new medium. At lower inoculum densities, however, the heat production rate was so low, below the detection limit of the calorimeter. This result is in agreement with that obtained by James (1987) for bacterial cells where at least 10^5-10^6 cells mL^{-1} were needed in the inoculum to obtain a signal significantly greater than the noise level of the calorimeter. The minimum number of cells detected by a calorimeter may depend on the kind of cells, the growth phase of the cells and the type of calorimeter in use. This is due to the fact that various types of cells at different phases of growth may have variable heat production rates, and various calorimeters have different sensitivity values. Calorimeters with higher sensitivity values are of value for the detection and study of cells at lower cell densities such as suspensions of cells available in small quantities (eg. tumour cells available from patients) and detection of contaminations in foods and other materials. Thus, the long calorimetric lag phase at lower cell densities ($< 10^6$ cells mL^{-1}) does not represent the biological lag phase alone but is introduced due to the insensitivity of the calorimeter. As a result the position of the P-t curve is shifted to the right at lower cell densities.

The variation in the lower inoculum densities used did not affect the shape of the P-t curve, Q_t and cell density at the peak. This is very important for the identification of microorganisms by their P-t curves from different samples such as dairy products where the inoculum size may not be strictly controlled. In addition, effect of antibacterial agents on the

P-t curve can be investigated without the need to strictly control the inoculum density, provided that they are added at a specific point in the curve.

Since the P-t curve is a result of the metabolic behaviour of the organism under a given experimental condition it does not change unless there is a change in the physical or chemical environment and/ or the genetic make up of the organism. As a result the P-t curve can be used as a "finger print" to identify an organism. Identification of microorganisms by their P-t curves was achieved by Boling *et al.*, (1973); Lamprecht, (1984); Russel *et al.*, (1975a and 1975b); Schaarschmidt and Lamprecht, (1976).

Comparison of the P-t curve with Q_t and other reflections of the metabolic events in the fermenter such as OD, oxygen consumption rate, oxygen concentration in the flow line and heat production rate per cell (Fig. 8 A - C) showed that both of these curves entered the stationary phase at almost the same time. This confirmed that the P-t curve is a true picture of the metabolic events in the fermenter under the chosen experimental conditions and didn't incorporate experimental artifacts due to separation of the fermenter from the calorimeter. Hence, the pumping rate (100 mL h^{-1}) is sufficiently high to take samples from the fermenter to the calorimeter rapidly, 1.42 min, so that depletion of oxygen may not occur in the flow line to cause a drop in the P-t curve, while metabolism continues in the fermenter at least at lower cell densities. This pumping rate is also sufficiently low that a sample takes time, long enough, to travel from fermenter to calorimeter that thermal equilibration of the suspension to the working temperature of the calorimeter is achieved before it enters the calorimetric vessel.

After attaining the stationary phase, the curve for Q_t showed slight increase with time whereas the others didn't. This is because Q_t is an integral of time and as far as there is metabolism, though at a very low rate, it continues increasing. Though OD is also an integral of time, it entered the stationary phase. This may be due to the absence of cell division or due to death of some cells. The other features of the culture mentioned, which are time derivatives, remained constant for some time.

The concentration of oxygen in the flow line, though it was highly reduced, didn't drop to zero (Fig. 8B). This showed that aerobic metabolic rate might have been reduced due to lowered oxygen concentration in the flow line, but could not be completely blocked due to absence of oxygen since there was some. This may be one possible reason for why the P-t curve didn't drop to zero after it returned to a minimum value after the peak.

The C/R ratio remained constant in the lag and exponential phases and increased after the first peak (Fig. 8B). This may be due to the presence of anaerobic metabolism, in addition to aerobic respiration, introduced due to reduced oxygen concentration in the flow line.

The heat production rate per cell was very low at the lag phase. Since the inoculum was taken from cells grown for 24 hours, most probably after entrance in the stationary phase, and transferred to a new environment they need time to synthesize cell materials. Hence anabolism might have dominated at this phase and catabolic heat release was very low. After the lag phase, however, catabolism might have dominated and hence the heat released per cell increased drastically (Fig. 8C).

Addition of EEP at different phases of growth showed an abrupt change in the heat production rate (Fig. 9 A - D). This result was in accord with the proposal of Beezer and Chowdhry (1980) that the presence of metabolic modifiers in a medium can significantly affect the appearance of a P-t curve. The curves clearly show that the effect of EEP is bactericidic, not bacteriostatic, because the heat production rate decreased drastically to a certain minimum point as soon as EEP was added. If the action was bacteriostatic the culture would be expected to continue metabolism at a fixed rate and hence a plateau in the P-t curve would have been achieved which may drop later on due to nutrient limitation or other factors (Buckton, 1995).

The action of ampicillin is bacteriostatic at lower concentrations and bactericidic at higher concentrations (Fig. 13). This is because the P-t curve remained at a plateau phase at lower concentrations and dropped down with increasing concentrations. The drop in the P-t curve might be due to killing of cells which increased with concentration. Even for concentrations exhibiting inhibitory effects, the extent of inhibition was higher at higher concentrations and hence the plateau phase lied below the plateau of the P-t curves at lower concentrations.

For all propolis samples used, the time per assay (i.e. the time from the point of addition to the minimum in the curve) was not greater than 10 minutes. Since the time to reach the mid exponential phase was *ca* 7 hours, the total assay time was therefore 7 hours and 10 minutes unless one incubates the culture to see further interactions. This assay time is very short as compared to the assay time for the agar well diffusion assay, which needs 12 - 48 hours or more (48 hours in the present work with the agar well diffusion assay method). In the case of ampicillin calorimetry, however, the assay time was longer than for EEP due to the

time needed for action to be observed on the P-t curve. Even in this case the assay time for the calorimetric method was shorter than that for the agar well diffusion method.

In addition to a shorter assay time, the calorimetric method has a higher reproducibility or precision (Beezer and Chowdhry, 1980). This is because a drug can be added at a specific point in the P-t curve, which under certain experimental conditions, always corresponds to a certain cell density; hence a highly reproducible experiment can be achieved. In addition, calorimetric methods do not require a large number of observations. On the other hand, classical microbiological assay methods suffer from the use of unstandardized inocula leading to conflicting results between or within laboratories and require large numbers of observations for satisfactory results and are labour intensive (Lightbown *et al.* 1963).

When EEP was added to the fermenter in the phases of growth at and before the main peak a drop in the P-t curve was observed (Fig. 9 A, B and C). This could be due to killing of cells and hence loss of heat production. Addition of EEP to the fermenter after the curve has dropped to a minimum value, after the main peak, resulted in an increase in power output (Fig. 9D). This could be accounted for by the fact that addition of EEP at this phase killed some cells thereby resulting in the reduction of cell density. Consequently the pressure imposed by high cell density on the culture, such as oxygen limitation, was released. As a result each cell might have started to metabolise and heat production rate per cell increased. With increasing concentration of EEP, the amount of cells killed increased, the unkilled cells getting much space and oxygen. As a result heat production rate per cell of the survivors, increased with increasing concentration of the antibacterial agent. In all cases tested (Fig. 9D) the P-t curve after being raised to a high value due to addition of EEP, soon returned to a minimum value with a slope comparable to the fall of the curve from the main peak in the control experiment. The effect of overcrowding of cells, as was in the main peak, might have operated here due to metabolism and growth of the survivors.

EEP killed cells as soon as it was added. However, ampicillin needed some time (2 - 3 hours) for its effect to be seen. Even after this time it did not kill immediately, but inhibited. Ampicillin first showed an inhibitory effect represented by a plateau phase of the P-t curve, whose length is inversely proportional to concentration, and then started killing. At lower concentrations, such as 0.072 mg mL^{-1} no killing effect was observed.

From the results of the agar well diffusion assay it was impossible to tell whether the effect of the antibacterial agent is bacteriostatic or bactericidic. It could only show whether the

chemical has an antibacterial effect or not. This is because there is no way of recording the kinetics of action of the antibacterial agent with the incubation time on the plates and the clear zone does not tell any thing about the mechanism and kinetics of action.

Application of EEP obtained from different geographical origins at the mid exponential phase rendered similar patterns of response (Fig. 10 A - D). Depending on type and concentration of EEP, the P-t curve dropped to a minimum value, as soon as EEP was added in the fermenter. This suggests that all EEPs exhibit bactericidic activities. The drop of the curve from the point of addition of EEP to the minimum point after addition is directly proportional to the amount of cells killed which again is directly proportional to the concentration of EEP added. In other words, the position of the P-t curve relative to the baseline after the curve has dropped due to addition of EEP is an indication of the amount of metabolizing cells mL^{-1}. With increasing concentration of EEP added the density of metabolizing cells is reduced and hence the curve approaches the baseline more and more.

After the P-t curve has dropped to a minimum value due to addition of EEP, a constant metabolic rate was observed which was represented by a horizontal line at higher concentrations and the trough at lower concentration. This phase could be a result of inhibition of growth of the survivors while having a constant metabolic rate. Determined by type and concentration of EEP, this inhibition phase lasted for some hours, after which growth was resumed. Prop1 and prop2 exhibited a higher inhibition and hence growth was not resumed at concentrations ≥ 0.6 mg mL^{-1} (Fig. 10 A and B). Even if the two samples showed the same pattern at these higher concentrations, prop2 exhibited longer period of inhibition time than prop1 at lower concentrations (< 0.6 mg mL^{-1}) where growth was resumed after sometime. At a concentration of 0.8 mg mL^{-1}, tincture showed the least inhibition time and hence growth was resumed soon. The resumption of growth after treatment with EEP may be achieved in two ways: (i) Despite the huge mass of inhibited cells, some cells might have not been inhibited by EEP. These cells might have been present at very low cell density metabolising and dividing continuously in addition to a constant heat production by the inhibited cells. The heat output by the actively metabolizing cells might have been very small as compared to the constant heat production rate by the inhibited cells to cause a change in the power output of the calorimeter. When the heat production rate by these cells bypasses the detection limit of the calorimeter, the P-t curve starts to rise and a second P-t curve is established. (ii) The inhibition on some cells might have been weak enough and lasted for few hours and/ or

minutes. Hence cells were released from inhibition and resumed normal growth and metabolism. The duration of inhibition was dependent on the type and concentration of EEP used. Since EEP is composed of lot of compounds, cells might have been inhibited by various chemicals. Hence the severity and duration of inhibition may depend on the type and concentration of different compounds present in EEP. As a result cells may be released from inhibition and resume normal growth and metabolism at different times of the incubation period to establish a second P-t curve.

Since propolis from different regions have different constituents at different proportions (Ghisalberti, 1979), the mechanisms of antibacterial action by different EEPs may not be the same, even if they kill/ inhibit bacterial cells. That is why, for example, 0.8 mg mL^{-1} EEP of tincture and prop2 even if they result in a drop of the P-t curve by 0.43 mW mL^{-1} and 0.44 mW mL^{-1}, respectively, growth was resumed with in 8.25 hours for the former and no growth was resumed for the latter with in 15 hours (Fig. 10 B and D), showing that the severity of inhibition exhibits variation.

The dose-response curves of calorimetry for all EEP were peculiar of antibacterial agents obtained by other methods, too. Comparison of activity based on the MIC values showed that prop2 and prop3 posses higher activity than the other two. From subsequent activity values in the dose-response curve and the time needed for the resumption of growth after treatment, prop2 showed higher activity than the others.

EEP possesses water soluble and insoluble components which are both soluble in alcohol. Addition of water to EEP resulted in the precipitation of water insoluble components. Both the water soluble and insoluble components of EEP were bactericidic (Fig. 12 A - D). The antibacterial effect of EEP was greater than even the sum of two of its components at a concentration of 1 mg mL^{-1} for each propolis except for tincture where the effect of the latter was slightly greater than the former. This may suggest that the two components of EEP may have a synergistic interaction. After fractionation, higher antibacterial activity was observed for the water insoluble components showing that the most active antibacterial compounds are present in this fraction. The activity of the water soluble components, as evaluated by the drop in the P-t curve and resumption of growth after the fall of the P-t curve, was lower than EEP and the water insoluble components. MIC values for water soluble components were higher than the other two and have to be applied at higher concentrations to kill cells. The water insoluble components, however, like EEP exhibit activity even at lower concentrations.

Since EEP from different regions have different constituents in both the water soluble and insoluble components, the variation in activity of these components from different propolis samples is inevitable. Comparable antibacterial activities of a given fraction (eg. water insoluble fraction) from different propolis samples may not necessarily mean that they have similar composition. It rather shows that the total sum of the activities of all components in the fraction from different propolis samples are equal.

The synergistic interaction between the water soluble and insoluble components was higher in some types of propolis than in others. For example, prop3 exhibited a higher synergistic interaction between the water soluble and insoluble components than for the other propolis samples. But tincture showed the lowest synergistic interactions of all and hence the effect of EEP was not significantly greater than either of the two, especially the water insoluble component.

Results from the agar well diffusion assay showed the same pattern as the calorimetric results in that the antibacterial activity for a given propolis was in the order of EEP > water insoluble component > water soluble component. Differences in activity among different EEPs were observed. Maximum activity was seen for EEP of prop2, with a killing zone of 4.65 mm at a concentration of 10 mg mL^{-1}, which is in agreement with the calorimetric results. The water soluble and insoluble components of tincture showed higher activity than the same components from other propolis.

The saturation of the dose-response curve for prop3 at a concentration of $\geq$ 5 mg mL^{-1} (Fig. 14C) may due to the precipitation of the water insoluble components in the agar matrix and blockage of the diffusion path. As the diffusion path is closed, further diffusion is terminated and hence compounds remain undiffused in the well. Precipitations were observed in the agar wells even at lower concentrations. The amount of precipitate at each concentration was higher for prop3 than for EEP from other propolis samples. This may due to the fact that prop3 had a higher proportion of water insoluble to water soluble components (0.0772g: 0.028g per 0.1 g of EEP) (Fig. 5).

The MIC values for all EEP by the agar well diffusion assay method were lower than the MIC values obtained by calorimetry. This may be due to the lower sensitivity of the calorimeter to detect heat loss as a result of killing of very few cells at very low concentrations of EEP. But in case of the agar well diffusion assay method, killing of even very few cells in the vicinity of the well makes the area free of any bacteria and hence further growth is

prevented; as a result a clear zone develops as far as there is killing and/ or inhibition. Therefore, even if there is killing and / or inhibition in the calorimetric experiment, at lower concentrations of antibacterial agents the effect on the P-t curve is observed as far as the heat lost is above the detection limit of the calorimeter. This problem can be solved by employing highly sensitive calorimeters which can detect heat production by few cells.

6. CONCLUDING REMARKS

Investigation of bacterial growth and metabolism can be done by using a flow calorimeter. At an optimum pumping rate the calorimetric signal is a true picture of the metabolic events in the fermenter. Hence, metabolic patterns and effects of antibacterial agents on metabolism can be investigated calorimetrically.

Insensitivity of the calorimeter for heat production in the presence of $\leq 10^6$ cells mL^{-1} is the major drawback of the calorimeter for the investigation of cellular metabolism at low cell density. Lower sensitivity of the calorimeter contributes to the undetectability of effect of lower concentrations of antibacterial agents on microbes. As a result the MIC values obtained by the agar well diffusion assay method for all propolis samples and ampicillin are lower than that obtained by microcalorimetry. Even if the calorimeter is less sensitive to detect effect of lower concentrations, it reveals the mode of action (i.e. whether bacteriostatic or bactericidic) of an antibacterial agent. However, the agar well diffusion assay method gives no information as to the mode of action of an antibacterial agent. Since spectrophotometry can not be used for the investigation of the mode of action of propolis on bacteria, calorimetry is the best method suited for this purpose.

Propolis samples of different origin show the same yield (w/w) upon extraction with 70% methanol. This may be due to the fact that propolis has the same basic make up regardless of its origin. The types of compounds present in propolis depend on its origin and hence varying proportion of water soluble and insoluble components are available in different propolis samples.

The higher antibacterial activity of EEP as compared to its water soluble and insoluble components when alone is an indication of synergistic interaction of the latter two in the former. The extent of synergistic interaction between the water soluble and insoluble components in EEP exhibits variation in different propolis samples. This is due to the fact that propolis from different geographical localities possess various compounds and hence the extent of interactions are quite different.

Since propolis kills cells as soon as it is added to the culture, it can be used to kill cells and sterilize a surface within a few minutes/ seconds. The use of propolis for medical applications may help to alleviate the economic constraints of the poor to buy drugs and is also a "back to nature" trend reducing the side effects of most drugs.

Since propolis from different geographical localities was found to have antibacterial activity it may be used for medical applications irrespective of its geography of origin though the antibacterial effect showed slight variation from region to region. The use of natural preparations such as propolis in medicine, especially for developing nations, may help to reduce the amount of money spent to import drugs and hence could contribute in the development of a nation.

7. REFERENCES

Aga, H.; Shibuya, T.; Sugimoto, T.; Kurimoto, M. and Nakajima, S. (1994). Isolation and identification of antimicrobial compounds in Brazilian propolis. *Biosci. Biotech. Biochem.* **58**, 945-946.

Armstrong, G.T. (1964). Calorimeters in metabolic studies: A review. *J. Chem. Educ.* **41**, 297-321.

Azevedo, I.B.S.; Sampaio, R.F.; Montes, J.C. and Contreras, R.L.L. (1986). Treatment of decubitus scabs with propolis. *Revista Brasileira de Enfermagem* **39**, 33-37.

Azuma, I. and Jolles, G. (1987). Immunostimulants. Now and tommorow. In: *Proc. French-Japan Joint Conference on Immunomodulators, Paris, 1986.* Azuma, I. and Jolles, G. (eds.). Springer-Verlag, Berlin, pp. 85-120.

Bäckman, P. (1991). Heat production in cellular systems. A thermochemical and methodological approach. *PH.D. thesis.* Lund University, Lund, Sweden.

Baldry, P.E. (1967). The battle against bacteria. Cambridge University Press. Brooke Crutchley, Cambridge.

Bankova, V.; Christov, R.; Kujumjiev, A.; Marcucci, M.C. and Popov, S. (1995). Chemical composition and antibacterial activity of Brazilian propolis. *Z. Naturforsch.* **50c,** 167-172.

Battley, E.H. (1987). Energetics of microbial growth. John Wiley & Sons. A Wiley-interscience Publication.

Beezer, A.E. and Chowdhry, B.Z. (1980). Microcalorimetric investigaitons of drugs. In: *Biological Microcalorimetry.* Beezer, A.E. (ed.) Academic press London. pp. 195-246.

Beezer, A.E. and Chowdhry, B.Z. (1981). Flow microcalorimetric bioassay of polyene antibiotics: Interactions with growing *Sacharomyces cerevisiae. Expermentia* **37**, 828-830.

Blaxter, K.L. (1978). The energy metabolism of ruminants. *Proc. Natur. Soc.* **37**, 1-3.

Boling, E.A.; Blanchard, G.C. and Russell, W.J. (1973). Bacterial identification by microcalorimetry. *Nature (London)* **241**, 472-473.

Brettel, R.; Corti, L.; Lmaprecht, I. and Schaarshmidt, B. (1972). Combination of a continous culture with a flow microcalorimeter. *Stud. Biophys.* **34**, 71-76.

Buckton, G. (1995). Applications of isothermal microcalorimetry in the fields of pharmaceutical sciences. *Thermochimica Acta* **248**, 117-129.

Buxton, A. and Fraser, G. (1977). Animal microbiology. Blackwell Scientific Publications. Oxford, London, Edinburgh, Melbourne

Calvet, E. (1948). Recent progress in calorimeters. *Seances. Acad. Sci.* **226**, 1702-1750.

Cliffe, A.J.; McKinnon, C.H. and Berridge, N.J. (1973). Microcalorimetric estimation of bacteria in milk. *J. Soc. Dairy Technol.* **26**, 209-210.

Crane, E. (1988). Beekeping: Science. Practice and World resources. Heinmann, London.

Derevici, A.; Popesco, A. and Popesco, N. (1964). Research on certain biological properties of propolis. *Annls Abeille* **7** (**3**), 191-200.

Derevici, A.; Popesco, A. and Popesco, N. (1965). Biological properties of propolis. *Revue. path.comp.* **2** (**1**), 21-24.

Dimov, V.; Ivanovska, N.; Bankova, V.; Manolova, N.; Nikolov, S. and Popov, S. (1991). Immunomodulatory action of propolis. Influence on anti-infectious protection and macrophage function. *Apidologie* **22**, 155-161.

Dimov, V.; Ivanovska, N.; Bankova, V. and Popov S. (1992). Immunomodulatory action of propolis: IV. Prophylactic activity against Gram-negative infections and adjuvant effect of the water-soluble derivative. *Vaccine* **10 (12)**, 817-823.

Dobrowolski, W.; Vohora, S.B.; Sharma, K.; Shah, S.A.; Naqvi, S.A.H. and Dandiya, P.C. (1991). Antibacterial, antifungal, antiamoebic, antiinflamatory and antipyrutic studies on propolis bee products. *J. Ethnopharmacol.* **35, 77-82.**

DSM, Catalogue (1989). German collection for microorganisms and cell culture. Braunschweig, Germany.

Errikson, R.; and Wadsö, I. (1971). Design and testing of a flow microcalorimeter for studies of aerobic bacterial growth. In: *Proceedings of the first European Biophysics congress.* 4. Broda, E.; Locker, A. and Springer-Leder, H. (eds.). Vienna: Verlag der Wiener Medizinische Akademie, pp. 319-327

Forstner, H. and Gnaiger, E. (1983). Calculation of equilibrium oxygen concentration. In: *Polarographic oxygen sensors: Aquatic and physiological applications.* Gnaiger. E. and Forstner, H. (eds.). Springer-Verlag, Berlin, Heidelberg, New York, pp. 321-333.

Ghisalberti, E.L. (1979). Propolis: A review. *Bee World* **60**, 59-83.

Ghisalberti, E.L.; Jefferies, P.R.; Lanteri, R. and Matisons, J. (1978). Constituents of propolis. *Expermentia* **34**, 157-158.

Gravelle, P. (1977). The microcalorimetric study of catalytic reactions. In: *Applications of calorimetry in life sciences. Proceedings of the international conference in Berlin,*

August 2-3, 1976. Lamprecht, I. and Schaarschmidt, B. (eds.) Walter de Gruyter, Berlin, pp. 315-322.

Greenaway, W.; Scaysbrook, T. and Whatley, F.R. (1987). The anasysis of *populus x euramericana*, and propolis by gas chromatography-mass spectrometry. *Proc. R. Soc. Lond.* **232**, 249-272.

Greenaway, W.; Scaysbrook, T. and Whatley, F.R. (1990). The composition and plant origin of propolis: A report of work at oxford. *Bee world* **71**, 107-118.

Hadden, J.W.; Spreafico, F.; Yamamura, Y.; Austen, K.F.; Dukor, P. and Masek, K. (1989). Immunomodulation through natural or synthetic substances. *Advances in immunopharmacol.* **4**, 55-80.

Hemminger, W. and Höhne, G. (1984). Calorimetry: Fundamentals and practices. Verlag Chemie, Darmstadt, Germany.

Hitchman, M.L. (1983). Calibration and accuracy of polarographic oxygen sensors. In: *Polarographic oxygen sensors: Aquatic and physiological applications.* Gnaiger, E. and Forstner, H. (eds.), Springer-Verlag, Berlin, Heidelberg, New York, pp. 19-30.

Hölzel, R.; Motzkus, C. and Lamprecht, I. (1994). Kinetic investigation of microbial metabolism by means of flow calorimeters. *Thermochimica Acta* **239**, 17-32.

Iannuzi, J. (1983). Propolis: The most mysterious Hive Element. A glimpse at the demand, etymology, sources, gathering, and hive use of beeglue. *Am. Bee J.*, **August**, 573-575.

James, A.M. (1987). Calorimetry, past, present and future. In: *Thermal and energietic studies of cellular biological systems.* James, A.M. (ed.). Wright, IOP Publishing Ltd. pp. 1-12.

Jones, M.N. (1979). Biochemical thermodynamics. Elsvier Publishers, Amsterdam, Netherland.

Lamprecht, I. (1983). Application of calorimetry to different biological fields and comparison with other methods. *Boll. Soc. Natur. Napoli* **92**, 515-542.

Lamprecht, I. (1984). Calorimetry in the identification and characterization of microorganisms. *Boll. Soc. Nature, Napoli* **93**, 570-583.

Lamprecht, I. (1985a). Application of thermoanalytical methods in biochemistry and biotechnology. *Thermochimica Acta* **23, 81-88.**

Lamprecht, I. (1985b). Direct and indirect calorimetry of medium sized animals. *Thermochimica Acta* **94**, 113-122.

Lamprecht, I. (1994). Calorimetric investigation around a royal heiroglyph. *Thermochimica Acta* **234**, 179-200.

Lamprecht, I.; Hemminger, W. and Höhne, G. (1991). Calorimetry in the biological sciences: Special issue. *Thermochimica Acta* **193**, 452-471.

Lavie, P. (1976). Relationship between propolis, poplar buds *(Populus sp.)* and *castoreum.* In: Lavie, P. (ed.) *Proc. of XXV Int. Beekeep. Congr. (Grenoble 1975).* Apimondia publ., Bucharest, pp. 227-231.

Lightbown, J.W.; Kogurt, M. and Uemeera, K. (1963). Bioassay of antibiotics by classical microbiological methods and by microcalorimetery. *Bull. Wld. Hlth. Org.* **29**, 87-100.

Marr, A.G. and Ingraham, J.L. (1962). Effect of temperature on the composition of fatty acids in *E. coli*. *J. Bacteriol.* 8 (4), 1260-1273.

Matsuno, T. (1995). A new clerodane diterpenoid isolated from propolis. *Z. Naturforsch.* **50c**, 93-97.

Mattiasson, B.; Larson, P.O. and Mosbach, K. (1977). Enzyme assay of cholesterol, glucose, lactose, and uric acid in standard solutions as well as in biological samples. *Nature* **268**, 519-520.

Möbus, B. (1972). The importance of propolis to honey bees. *Br. Bee J.* **100**, 198-199.

Monk, P.R. and Wadsö, I. (1968). A flow reaction microcalorimeter. *Acta Chem. Scand.* **22**, 1842-1852.

Motzkus, C.; Welge, G. and Lamprecht, I. (1993). Calorimetric investigation of phenol degradation by *Pseudomonas putida. Thermochimica Acta* **229**, 181-192.

Newell, R.D. (1980). The identification and characterization of microorganisms by microcalorimetry. In: *Biological microcalorimetry.* Beezer, A.E. (ed). Academic press, London, pp. 163-194.

Nikolov, N.; Ivanovska, N.; Manolova, N.; Bankova, V. and Dimov, V. (1991). Immunomodulatory action of propolis: Influence on antiinfectious protection and macrophage function. *Apidologie* **22**, 155-162.

Ogren, W. (1990). What in the world is propolis used for? *Am. Bee J.* **April,** 239-240.

Papay, V.; Toth, M.; Soltes, M.; Nagy, E. and Litkei, G. (1986). Isolated compounds from Hungarian propolis and *populi gemma. Stud. org. chem. (Amesterdam)* **23**, 233-240.

Poore, V.M. and Beezer, A.E. (1983). Heat conduction flow microcalorimeters. *Thermochimica Acta* **63**, 133 -140.

Randzio, S. and Suurkuusk, J. (1980). Interpretation of calorimetric thermograms and their dynamic corrections. In: *Biological microcalorimetry*. Beezer, A.E. (ed.), Academic Press, London. pp. 311-342.

Russel, W.J.; Farling, S.R,; Blanchard, G.C. and Boling, E.A. (1975a). Interim review of microbial identificaiton by microcalorimetry. In: *Microbiology*. Schlessinger, D. (ed.). Am. S. Microbiol, Washington, pp. 113-200.

Russel, W.J.; Zetller, J.F.; Blanchard, G.C. and Boling, E.A. (1975b). Bacterial identification by microcalorimetry, In: *New approachs to the identification of microorganisms*. Hegen, C.G. (ed.), New York, pp. 53-87.

Schaarschmidt, B. and Lamprecht, I. (1976). Calorimetric characterization of microorganisms. *Expermentia* **32**, 1230-1234.

Scheller, S.; Krol, W.; Swiacik, J.; Owerzarek, S.; Gabrys, J. and Shani, J. (1989). Antitumoral property of ethanolic extract of propolis in mice-bearing Ehrlich carcinoma, as compared to bleomycin. *Z. Naturforsh.* **44c**, 1063-1065.

Spink, C. and Wadsö, I. (1976). Calorimetry as an analytical tool in biochemistry and biology. *Meth. Biochem. Anal.* **23**, 1-159.

Strehl, E.; Volpert, R. and Elstner, E.F. (1994). Biochemical activities of propolis extracts. (III) Inhibition of dihydrofolate reductase. *Z. Naturforsch.* **49c**, 39-43.

Sturtevant, J.M. (1971). Application of thermoanalytical methods in biochemistry. Physical methods of chemistry. John Wiley & Sons.

Sunner, S. and Wadsö, I. (1959). Adiabatic calorimeters. *Acta Chem. Scand.* **13**, 97-123.

Thomson, W.McL. (1990). Propolis. *The Med. J. of Australia* **153**, 654.

Tian, M. (1923). Design of a thermopile heat conduction calorimeter. *Bull. Soc. Chem. Fr. Ser.* **4 (33)**, 427-454.

Tiefenbrunner, F. (1977). Microcalorimetric investigations of aquatic biotops. In: *Application of calorimetry in life sciences*. Lamprecht, I. and Schaarschmidt, B. (eds.). Walter de Gruyter, Berlin, pp. 305-314.

Tomas-Barberan, F.A.; Garcia-Viguera, C.; Vit-Oliver, P.; Ferreres, F. and Tomas-Lorente, F. (1993). Phytochemical evidence for the botanical origin of tropical propolis from venezuela. *Phytochemistry* **34**, 191-196.

Toshkov, A.; Dimov, V.; Denechev, V. and Vassilev, C. (1989). Immunomodulators in infectious diseases. State Publishing House Medizina Fiskultura, Sofia.

Volpert, R. and Elstner, E.F. (1993). Biochemical activities of propolis extracts. (I) Standardization and antioxidative properties of ethanolic and aqueous derivatives. *Z. Naturforsch.* **48c**, 851-857.

Volpert, R. and Elstner, E.F. (1996). Interactions of different extracts of propolis with leukocytes and leukocytic enzymes. *Arzneim.-Forsch. Drug Res.* **46(I)**, 47-51.

Wadsö, I. (1987). Calorimetric techniques. In: Thermal and energietic studies of cellular biological systems. James, A.M. (ed.). Wright, IOP Publishing Ltd. pp. 35-67.

Wadsö, I. (1990). Progress and problems in microcalorimeteric work on living systems. In: *experimental chemical thermodynamics, 2, Solution chemistry.* Marsh, K.N. and O'Hare, A.G. (eds.). *International Union of Pure and Applied Chemistry (IUPAC)*, pp. 13-30.

Wang, L.; Mineshita, S.G.I.; Shigematsu, T. and Matsuno, T. (1993). Antiinflammatory effect of propolis. *Jpn. J. Pharmacol. Ther.* **24**, 223-224.

Wright, H.N. and Montag, M. (1959). A text book of pharmacology and therapeutics. 7th edn., Saunders company, Philadelphia, London.