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Application of Isothermal Calorimetry to Monitor Microbial Growth and Metabolism in Soils and Fresh Food Products

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ABSTRACT

This PhD research project aims to verify the use of Isothermal Calorimetry (IC) as a valid technique to assess the microbial growth in different environments, in either liquid or solid state. In liquid stirred cultures every bacterium grows essentially in the same environmental conditions, and this simplifies significantly the study and characterization of the growth processes, while bacterial growth in solid media is much more difficult to study.
Isothermal calorimetry (IC) represents a powerful method to directly monitor the biological activity of such living systems since it provides direct quantitative information about the relevant energetics and kinetics.
In the first part of the research, we focused our attention on the study of two microorganisms involved in the oxidation/reduction of arsenic which is a ubiquitous contaminant related to natural weathering and industrial or agricultural activities and strongly affects the quality of cultivable soils used for the production of edible crops.
In this part of the study, calorimetric measurements are proven to be suitable to evaluate the toxic effect of arsenic on the growth of two microbial strains (*Achromobacter* sp. and As5-13 *Bacillus* sp. 3.2) isolated from soil and groundwater.
The toxicity and mobility of this element depend on chemical speciation and its oxidation state, and are specific for each microorganism, for instance inorganic arsenite As(III) is more toxic and mobile than arsenate As(V). Microorganisms can directly affect the redox balance As(V)/As(III) shifting it toward the species that can be more easily processed and/or absorbed by scavenger plants.
IC was applied in the first step to assess the effects of arsenic on the growth, metabolism and the As(V)/As(III) redox capability of *Achromobacter* sp. As5-13 strain, a well characterized microorganism isolated from an agricultural soil. Other parallel measurements were performed with conventional methods, such as turbidity or microbial count, and standard chemical analysis to determine the arsenic concentration.
In the second step IC and classical microbiological methods were applied to evaluate the influence of As(V) and As(III) on the growth and metabolism of *Bacillus* sp. 3.2 (isolated from groundwater) in different culture medium, namely, Tris Mineral Medium with Gluconate (TMMG) with the addition of yeast extract, and Tryptone Soya Broth (TSB).
The IC thermo-grams in combination with the microbiological counts and the chemical analysis data on the arsenic oxidation status during the microbial activity, permitted to discriminate the influence of arsenic on the energetic aspects of the microbial metabolism in conditions that mimic different kind of cultivable soils.
Regarding the second part of the research, we started exploring bacterial growth in solid state media choosing two different foods matrixes: carrots and corn salad.
In the first step we used IC to investigate the effect of different cuts on carrots (cylinders, sticks and juliennes) in order to monitor the kinetics of microbial growth and to simplify this research we selected juice carrot and *Pseudomonas fluorescens* sp., a well characterized microorganism isolated from carrots, as a model microorganism.
In the second step, IC studies were performed to assess the shelf life of corn salad, choosing three different temperatures of storage (5, 10 and 20°C), studying the kinetic evolution of chemico-physical (color, total phenols, moisture, pH variation and flow heat) and microbiological indices. We used for this study two different calorimeters in order to evaluate the influence of the temperature on the kinetics of microbial growth.
The results obtained in this project showed that calorimetric method used in combination with other relevant methods is a very powerful tool in studying the kinetic and metabolic growth of bacteria in different fields. Moreover, calorimetry assays, essentially consisting in on-line measurements, are very precise and easy to carry out in comparison with other methods currently used.

RIASSUNTO

In questo progetto di tesi di dottorato abbiamo utilizzato la Calorimetria Isotermica (CI) come metodica valutativa della crescita microbica in ambiente liquido e in ambiente solido. Nella fase liquida i batteri hanno una crescita omogenea in tutta la matrice garantendo una maggior facilità nello studio e dei risultati omogenei in tutto il campione; cosa che non avviene nelle matrici solide dove ci possono essere zone disomogenee con relativa differenza nella crescita microbiologica.
La calorimetria isotermica può fornire informazioni quantitative sull'energia sviluppata durante un processo biologico e la cinetica dello stesso processo; sia esso un evento enzimatico del substrato o dovuto al metabolismo batterico, oppure una commistione di entrambi.
Nella prima parte del progetto abbiamo focalizzato l'attenzione su due microrganismi coinvolti nelle ossido-riduzioni dell'arsenico; metallo inquinante presente nelle acque di scarico d'alcune attività industriali o agricole inquinando sia le falde acquifere che i terreni ad uso agricolo.
L'utilizzo della CI si è rilevato adatto per la valutazione dell'effetto tossico dell'arsenico sulla crescita dei due ceppi selezionati e isolati in precedenza (*Achromobacter* sp. As5-13 e *Bacillus* sp. 3.2).
La tossicità e mobilità di tale elemento dipende dalla speciazione chimica; per esempio la forma inorganica As(III) risulta maggiormente tossica e mobile rispetto all'As(V). I microrganismi possono facilmente influenzare lo stato d'ossidazione convertendolo nella forma più facilmente assorbibile dalle piante pulitrici.
Mediante la CI è stato valutato l'effetto dell'arsenico sul metabolismo e sulla capacità ossido-riduttiva del ceppo *Achromobacter* sp. As5-13 isolato da un terreno agricolo; contemporaneamente sono state eseguite le analisi convenzionali quali la misura della torbidità, la conta microbica e le analisi chimiche per valutare la concentrazione dell'arsenico.
Nella seconda fase sono state eseguite le analisi calorimetriche e classiche per valutare l'influenza dell'As(III) e dell'As(V) sulla crescita e il metabolismo del *Bacillus* sp. 3.2, isolato dalle falde acquifere, utilizzando diversi terreni di cultura come il TMMG (Tris Mineral Medium with Gluconate) con aggiunta di estratto di lievito e il TSB (Tryptone Soya Broth).
I tracciati calorimetrici in combinazione con le conte microbiche e le analisi chimiche dell'ossidazione dell'arsenico durante l'attività microbica, permettono di valutare l'influenza dell'arsenico sull'energetica metabolica microbica in diverse tipologie di suolo.
Per investigare la crescita microbica in sistemi alimentari freschi, seconda parte di questo lavoro, abbiamo scelto di utilizzare matrici solide ossia carota fresca e insalata di varietà "Valerianella" (quarta gamma).
In particolare, abbiamo utilizzato la CI per misurare l'effetto di diversi tipi di taglio nella carota (cilindro, bastoncino, Juliennes) sulla cinetica della crescita microbica. Prove comparative sono state eseguite anche su del succo di carota; in questa fase abbiamo utilizzato il ceppo *Pseudomonas fluorescens* come microrganismo modello il quale è stato isolato dalle stesse carote utilizzate per le altre prove.
L'insalata è stata utilizzata per uno studio di shelf-life in prodotti di quarta gamma, simulando tre differenti temperature di conservazione (5, 10 e 20°C), analizzandone le diverse cinetiche di crescita microbiologica e l'evoluzione dei differenti parametri chimico-fisici (colore, polifenoli totali, umidità e pH). Utilizzando due diversi calorimetri isotermi abbiamo potuto eseguire le analisi alle diverse temperature di stoccaggio, ottenendo direttamente i risultati calorimetrici durante i vari tempi di conservazione.
Dai dati raccolti durante questo lavoro di tesi risulta che, il metodo calorimetrico isotermo, unito ad altre metodiche più classiche, si è rilevato un valido sistema per lo studio rapido e preciso della cinetica e del metabolismo coinvolto nella crescita microbica in matrici differenti.
La calorimetria permette analisi dirette e continue che sono molto precise e facili da ottenere rispetto alle analisi microbiologiche classiche.

PREFACE

In nature, all physical, chemical, and biological processes produce or consume heat. Processes that produce heat are called exothermic and processes that consume heat are called endothermic.
Devices that measure heat transfer are called calorimeters. One peculiar kind of calorimeter is the isothermal calorimeter. The results of calorimetric measurements should be interpreted in the framework of a thermodynamic approach so as to be used in a broader perspective.
The term "isothermal calorimeter" is commonly used for particularly sensitive calorimeters, which are used in experiments where the temperature remains constant. The sensitivity of such instruments is typically in the range of one microwatt or even better (Wadsö, 2001). These kinds of instruments allow determination of either kinetic or thermodynamic parameters from the power-time curve. These parameters include enthalpy change, Gibbs energy and entropy change (Yang el al., 2005).
Over the last decade, there has been an increase of the use of isothermal calorimetry in the biosciences. Isothermal Calorimetry (IC) can be used to assess microbial growth and metabolic activity (Barros et al., 2007). Similarly in environmental science, isothermal calorimetry has been shown to provide a valuable insight in soil science or in geo-microbiology. Most biochemical reactions in soil are catalyzed by enzymes. The microcalorimetry method would facilitate further understanding of the thermodynamics and kinetics of enzymatic reactions in the soil system. Soil microbial activity is an ideal bio indicator of soil pollution, which can be monitored by the microcalorimetry method. For these reasons the application of the method is expected to be widespread in the future. (Rong et al., 2007).
In the case of microbial growth, the calorimetric experimental data must be converted into biologically meaningful parameters, like growth rate, lag phase, or maximum growth (Braissant et al., 2013). In this study we review the various approaches used for such conversion, trying to highlight the advantages, the drawbacks and underlying assumption of each approach.
A modern calorimeter can detect the heat flow related to the metabolism of as few as 10000 per mL active bacterial cells, and can be used for different kinds of investigations: e.g.,
Medical applications: through the rapid detection of bacterial infection or contamination, determination of inhibitory effects and/or the minimal inhibitory concentration for different antimicrobial compounds and microorganisms;
Environmental microbiology: by directly assessing bacterial activities;
Soils application: by detecting and quantifying the microbial activities, monitoring of organic pollutant toxicity or degradation and assessing the risk associated with heavy metals contamination (Braissant et al., 2010).
In this thesis, we address the use of IC as an innovative tool for direct monitoring different aspects of the growth of microbial populations by using relevant case-studies that are representative issues of both the environmental and the food fields. Namely, the first case-study, i.e., the microbial growth in arsenic contaminated soils, regards a prevailing environmental issue, whereas the second and third case-studies, i.e., the microbial spoilage of two different food matrices, regard a capital challenge of the food industry: the shelf-life extension of the fresh produce and the reduction of post-harvest losses.

Case-study 1: microbial growth in arsenic contaminated media.

The environment concern on arsenic contamination has increased in the past few decades due to the increased awareness of its risk to plants, animals, and human health.
Arsenic can enter into soils, aquifer sediments and drinking water through various pathways, most of the time as insecticide, herbicide, and fungicide production (Sviridov et al., 2012; Gomila et al., 2011).
Although arsenic is a well known toxicant to most eukaryotes and prokaryotes, some prokaryotes such as *Alcalilimnicola halodurans*, *Halobacillus* sp., *Bacillus halodurans*, Arsenite-oxidizing bacterium

and *Bacillus arseniciselenatis* have evolved and survive under arsenic presence by their unique biochemical or physiological mechanisms (Oremland, 2004).
This chemical element is ubiquitous in soils and its concentrations range naturally from 1 to 40 mg arsenic kg^{-1} in uncontaminated soil, but can reach much higher levels in contaminated soils (Mandal, 2002). Arsenic mainly occurs in two inorganic forms, arsenite As(III) and arsenate As(V), both of which are highly toxic. Either forms are found in the environment, although As(III) is generally found in reducing environments, while As(V) predominates in well-oxidized conditions (Ackermann, 2008).
Arsenic toxicity and mobility depends on chemical speciation and the oxidation state (Thomas, 2001), inorganic species being more toxic and mobile than organo-arsenic species, whereas inorganic arsenite [As(III)] is more toxic and mobile than arsenate [As(V)] (Pontius, 1994). Squibb and Fowler found that As(III) was 10 times more toxic than As(V) and 70 times more toxic than monomethylarsonic acid [MMA(V)] and dimethylarsinic [DMA(V)] (Squibb & Fowler, 1994). In comparison to other species, the mobility of As(III) is very high because it is present as the neutral H_3AsO3 (Ascar et al., 2008). However, the available information on arsenic behavior in agricultural soils is limited compared to groundwater.
Accordingly, it is obvious that arsenic distribution and toxicology in soils has become a serious issue.
The metabolism of microorganisms influences the bio-geo-chemical cycle of As, affecting both its speciation and toxicity. The tendency for As(III) to mobilize into the aqueous phase results from the differences in the sediment sorption characteristics of As(V) and As(III) (Smedley & Phung, 2002). Bacteria develop different arsenic resistance mechanisms that can be divided into two basic categories consisting either of redox reactions that conserve the energy gained for cell growth or detoxification reactions through the ars operon genes (Mukhopadhyav et al., 2002; Oremland et al., 2005; Smedley & Phung, 2005).
Soil microorganisms affect As mobility and availability to the plant: they produce iron chelators and siderophores, reduce soil pH, and/or solubilize metal-phosphates (Abou-Shanab et al., 2003; Lasat, 2002), there by modifying the efficiency of the accumulation processes.
In recent years, calorimetry has been extensively used in studying the interaction between heavy metals and microorganism (Barja et al., 1997). Being a universal, integral, nondestructive, and highly sensitive tool for many biological investigations, the isothermal calorimetric technique directly determines the biological activity of a living system and provides a continuous measurement of heat production, thereby giving both extensive qualitative and quantitative information (Critter et al., 2004; Yao et al., 2005; Salerno-Kochan, 2010). Thermogenic curves obtained from calorimetric measurements can reveal temporal details about microbial metabolism not observable by other methods (Wadsö, 1995).
This PhD thesis reports the study of two bacterial species, *Achromobacter* sp. As5-13 and *Bacillus* sp. 3.2 (identified by the Laboratory of microbiology in Milan University, Dott. Anna Corsini*)*, that represent models of contaminating pathogens in soils and groundwater (Haman, 2011).
Achromobacter sp. As *5-13,* strain isolated from an aged arsenic-contaminated soil was used to screen for its limits to be strictly aerobic, the cells are negative straight rods and are usually found in water (freshwater and marine) and soils.
Bacillus sp. is widely distributed in nature and putrefactive organisms. Having become the paradigm for Grampositive bacteria, *Bacillus* sp. is an ideal experimental system for studying gene regulation, metabolism, and cell differentiation in view of the related microorganisms such as the human pathogen *Staphylococcus aureus* (Wolf et al., 2007). Therefore, the study of the toxicology of As(III) on the *Bacillus* sp. *3.2* strain is not only significant for understanding biological processes but also useful for deciphering environment Pathways. In this investigation, *Bacillus* growth in two different culture media (TMMG and TSB) was monitored continuously and the toxic effect of As(III) was measured calorimetrically using a DAM calorimeter. Simultaneously, the change of turbidity of selected microorganisms was measured, with the aim of verifying results obtained by calorimetry.

Case-study 2: microbial growth in minimally-processed carrots.

IC was also used to monitor the microbial spoilage of fresh food vegetables products and assess their shelf life in parallel with other chemico-physical analysis (Riva et al., 2001).
Recently, isothermal calorimetry is becoming a quite commonly used quantitative analytical technique for studies of fundamental biology, (Gustafsson, 1991; Kemp, 2000; Lamprecht, 2003) and in some cases become the connecting link between the food-related microbiological studies with a few studies of vegetable tissue respiration. Used principally in physics, chemistry or biology laboratories, it can provide real time informations about the yield, growth rate during a calorimetric measurement of a specific biological process, as heat flows through all materials one can monitor processes taking place inside opaque materials and packages.
It is a non-destructive technique and an interesting tool for looking at kinetics of all types of reactions and processes. This use is based on the fact that the thermal power P (W) measured is related to the rate ν of the process studied, and the corresponded integral i.e. the overall heat Q (or ΔH) produced, can be related to the overall process in term of microbial growth and overall metabolic activity (Gomez & Sjöholm, 2001).
Food is a complex matrix where a lot of process are evolving in the same time for this the total enthalpy is the sum of the enthalpies of all the individual processes, even if enthalpy change and rates are not as easily defined as for simpler reactions, can be measured thermal powers (and heats) can still be used to model kinetics of, e.g., degradation processes (Hansen, 2000).
The potential of isothermal calorimetry as an analytical tool to provide an integrated view of the effect of different processing steps on the quality and shelf life of processed fresh fruits and vegetables. (Franzetti et al., 1995; Riva et al., 1997), such as peeling, washing, slicing or shredding which create practical problems regarding self-life, safety and packaging and cause food deterioration (Barry-Ryan & O'Beirne, 1997) was so far assessed.
Deterioration due to microbial spoilage is usually caused by the autochthonous microbial population or by contamination during the processes experienced by the product, physiological ageing, biochemical changes and loss of nutritional quality. Physiological ageing usually implies a generalized loss of quality and can often be related to tissue death. It is driven by respiration, transpiration and other metabolic processes (Barry-Ryan & O'Beirne, 1997).
The predominant microflora of fresh and leafy vegetables is formed by *Pseudomonas* and *Erwinia* sp. An increase in the CO_2 concentration in the package will favor the development of different microflora, such as lactic acid bacteria (Ahvenainen, 1996), after the processing. At the same time the O2 concentration decreases resulting in a reduction in the respiration rate of the product (Kader & Saltveit, 2003). Production of CO_2 and consumption of O_2 can be detected by means of isothermal calorimetry as heat produced (Hansen et al., 1997).
All living systems produce heat as a result of their metabolism. This heat can be detected directly and continuously in calorimeters and used to assess the extent of metabolic activity, such as respiration and reaction to wounding stress (Gomez et al., 2004), or to monitor the microbial growth in fresh vegetables (Riva et al., 2001; Alklint, 2003). Heat production by microorganisms growing in food stuffs is well documented in the literature (Alklint, 2003; Riva et al., 2001).

In the first step of this research we investigated the relation between the degree of wounding and the microbial growth in carrots. A relation between the wounding intensity and susceptibility to spoilage by anaerobes can be established if the samples are introduced in the calorimeter ampoules long enough to cause oxygen depletion. In our case, this was found to be closely associated with the increase in the surface area of the samples.
The most common responses to wounding may include an increase in respiration rate, ethylene production, quality changes, and synthesis and/or loss of phytochemicals (Yang & Pratt, 1978; Kahl & Laties, 1989; Kader, 1992; Brecht, 1995; Tapadia at al., 1995; Saltveit, 2000).

Carrot with different degrees of wounding, reproduced with different cutting shapes, namely, fresh-cut cylinder, sticks and julienne, were used to understand the effect of wounding on the metabolism of the microbial population.
The carrot juice, produced from macerated carrots and separated from the pulp, hosts a residual microbial flora that is responsible for aerobic and anaerobic reactions. In the present work, the related microbial metabolism was monitored with isothermal calorimetry investigations.
Minimally processed fresh fruits and vegetables are good media for the growth of micro-organisms (Nguyen-the & Carlin, 1994) that affect the shelf-life (SL) of these products.
A fresh processed product is frequently less contaminated than the raw material, although the relevant microbial flora remains practically unaltered. The microbial flora in the carrot juice is thus likely to be similar to the flora on raw carrots, the spoilage of which (either shredded or sliced) was found to be associated with lactic bacteria (Nguyenthe & Carlin, 1994).
Fresh carrot juice is primarily spoilt by microorganisms, and their growth will determine the SL of the product. Monitoring the SL progress requires quick, reliable and, possibly, inexpensive microbial analyses.
The major drawbacks of traditional plate counting methods are the high costs of material and labor and the several day incubation. It is therefore of interest to exploit alternative methods. A simple and commonly used method is to monitor the pH, as it is changed by microbial metabolism. This is a rapid, but not very specific method, since other reactions may also affect pH. Another method is Isothermal Calorimetry (IC), where the heat produced in a sample is measured at constant temperature. The thermal power, P (W), is measured over time and integrated to find the heat, Q (J). The total amount of heat generated is related to the processes taking place in the material (Wadsö, 1997).
Once the pH of the culture medium is kept constant, the results obtained with this technique were found adequate to study the microbial activity in complex systems (Barja et al., 1997). For this reason IC investigations on microbial systems have been conducted both at the fundamental level and in several applied areas.

Case-study 3: shelf life of ready to eat salad.

Isothermal calorimetry has been used to follow microbial growth in different types of food and has been correlated to the shelf life of industrial whole eggs, fresh milk and carrot convenience salads that are usually stored in a refrigerated environment (Riva et al., 2001). The choice of the refrigeration temperature is of critical importance to maintain the quality of the packed product, since the temperature affects the conditions of the microbial growth and the composition of the overall microbial flora, according to the different susceptibility of the different strains hosted in the food (Firstenberg-Eden & Tricario, 1983; Garcia-Gimeno & Zurera-Cosano, 1997). The selection of the most adequate storage conditions is therefore crucial. In this perspective the calorimetric investigations can be of help as long as they allow the assessment of the kinetic parameters of the microbial spoilage process at various temperatures.
Generally many other experimental methods used to detect the quality changes provide an indirect information about the changes triggered by the storage conditions, or, more rarely, some information about the transformation mechanism, while very seldom they allow the kinetic parameterization of the food quality (Riva, 1997, Riva et al. 2001; Lampi et al., 1974; Iversen et al., 1989; Barja et al., 1997; Cliffe et al., 1973).
Shelf-life of fresh cut vegetables can be predicted either controlling the driving agents (growth of microbial populations, enzymatic activities, concentration of reactive compounds) or monitoring their effects, like changes in pH, color, texture, nutritional value, and presence of peculiar compounds. In the present study isothermal calorimetry was chosen to monitor the growth continuously in samples of

fresh cut corn salad that had been stored at various controlled temperatures, namely, 5, 10 and 20°C, which reflect a range of storage conditions that can be practically experienced by the product.
Plate counts and other data, namely, changes of pH, color and/or texture, obtained from separate batch cultures stored at the three selected temperatures, allowed complementary information to confirm the result of IC and suggest some interpretation.

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1. ISOTHERMAL CALORIMETRY AND MICROBIAL GROWTH.

1.1 DESCRIPTION OF MICROBIAL GROWTH

Microbial growth is a biological process of both the increase in number and the increase in mass of bacteria. Growth has three distinct aspects: biomass production, cell production, and cell survival. Cell production is contingent on biomass production; the biomass growth rate (dX/dt) is proportional to the biomass amount. The microbial growth is defined by the following relationship:

$$dX/dt = \mu * X \qquad (1)$$

This is valid since bacteria increase their number at a constant rate, in the batch culture, μ is the specific growth rate and is defined in the exponential growth phase and X is describing the biomass amount.
Integration of equation (1) leads to:

$$X = X_o e^{\mu t} \qquad (2)$$

were X stand for biomass, t is time and the subscript "0" indicates starting conditions.
The parameter μ represents the growth rate and has the dimension of reciprocal time (equation 3). μ is directly dependent on the concentration of limiting substrate as described by Monod's equation (equation 4) :

$$\mu = 1/X * dX/dt \qquad (3)$$

$$\mu = \mu_{max*} S/ S + K_S \qquad (4)$$

where S is the concentration of the limiting substrate, μ_{max} (h^{-1}) is the maximum specific growth rate and KS is the substrate specific constant, which describes the bacterial affinity towards the limiting substrate. KS is the Monod's constant that corresponds to the concentration at which the biomass growth rate is one-half of its maximum value.
If the substrate concentration is higher than K_S value (($S >> K_S$), then bacteria in the exponential growth phase are growing with the maximal possible growth rate ($\mu = \mu_{max}$). Taking into account Monod's equation the typical bacterial growth curve could be constructed (See Figure 1).
The specific growth rate μ depends directly on limiting substrate concentration and on process conditions (e.g., pH, temperature), concentrations of non-limiting substrates and/or metabolites formed. μ is positive in the case of microbial growth and negative in the case of microbial inactivation and prevailing death of cells (Van Impe et al., 2005).
Growth Kinetics of Pure Bacterial Culture in a Batch System, whether in a food product, normally passes seven phases:

1) Lag phase
2) Accelerating growth phase
3) Log (exponential growth) phase
4) Declining growth phase
5) Stationary phase
6) Death or declining death phase
7) Log death phase

But we can considerate for simplification the growth phases of a simple batch culture divided in three macro phases: lag phase (is the initial phases) during which represents the period (time) required for bacteria to adapt to their new environment, followed by an exponential growth during which the

bacterial cells divide regularly at a constant rate multiply and finally a stationary phase during which The number of cells remains constant perhaps as a results of complete cessation of division or the balancing of reproduction rate by an equivalent death rate. The maximum population density is reached as shown in Figure 1.

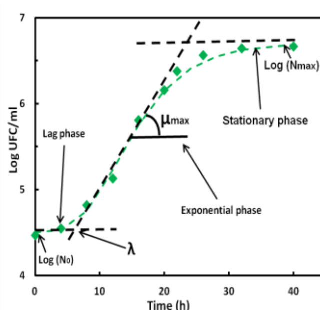


Figure 1.0. A typical growth curve of *Bacillus* sp. in a simple batch culture (adapted from van Impe et al., 2005).

During the lag phase of the bacterial growth cycle, the individual bacterial cells increase in size, but the number of cells remains unchanged, they are very active physiologically and are synthesizing new enzymes and activating factors.
Lag phase duration is influenced by inoculation rate (Robinson et al., 2001; Prats et al., 2006), pH (Francois et al., 2005; Ölmez & Aran 2005), inoculation temperature (Swinnen et al., 2004; Francois et al., 2005; Swinnen et al., 2005; Prats et al., 2006; Ölmez & Aran, 2005), limiting substrate concentration (Ölmez & Aran, 2005), "physiological state" of bacteria and difference between the pre- and incubation environmental conditions (Swinnen et al., 2004).
Lag phase is followed by exponential growth phase sometimes called the log phase or the logarithmic phase, during which the cells multiply exponentially. The number of cell produced during the exponential phase per time unit is proportional to the number of cells present. If growth is not limited, doubling of cells continues at a constant rate.
For this type of exponential growth, the plot of the logarithms of the number of cells versus time is a straight line. The slope of the growth curve is determined by the specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time. The growth rate is limited only by the bacteria's ability to process the substrate and depends on the growth conditions.
In order to determine what causes the end of the exponential growth - substrate concentration or formation of toxic products - the initial concentration of substrate can be changed. If the increase of initial substrate concentration causes the increase of the maximal bacterial concentration, then the limiting factor is the availability of the substrate. If the increase of the initial substrate concentration (C-source, N-source, vitamins, etc.) does not influence the final bacterial density, then the reason is most probably the formation of toxic products. This situation has been dubbed this "0-order growth" since the growth rate is constant and maximum.
The growth rate decreases in the stationary phase and the density of bacterial culture reaches its maximal value (Van Impe et al., 2005).

The experimental data may be rendered more meaningful and concise if they are analyzed in terms of the various growth kinetic parameters for this reason the bacterial growth curve could be described by four parameters:
- $y_{max} = \ln(X_{max})$, where X_{max} is the final bacterial number (CFU mL^{-1}),
- $y_0 = \ln(X_0)$, where X_0 is the initial bacterial number (CFU mL^{-1}),

• λ, lag phase duration (h),

• $\mu_{max} = \ln(2)/t_d$, where μ_{max} (h^{-1}) and t_d (h) are the maximum growth rate and the minimum duplication time, respectively.

In liquid cultures any bacterium is growing essentially in the same environmental conditions, and this simplifies significantly the study and characterization of the growth processes. Bacterial growth in solid states is a much more complicated phenomenon - solid matrices force bacteria to grow in colonies and thus diffusion of substrates and products, possible accumulation of toxic products in the colonies etc. lead to the different growth curves (laws). It has been reported in the literature (Wilson et al., 2002) that bacteria grow more slowly in structured systems than they do in broth. It has been proposed that growth in gelled systems is associated with additional stress on the organism. It has been also shown that the growth lag in solid media is longer than in broth (Malakar et al., 2002).

1.2 CALORIMETRIC METHOD

All life processes invariably and continuously dissipates Gibbs energy. As this is virtually always coupled with the continuous production and exchange of heat, calorimeters could be readily used to monitor life processes. Even though life processes are extremely complex and may be composed of thousands of individual metabolic reactions, calorimetric data obtained allow an interpretation of the overall metabolic process which can be described and thermodynamically treated as a simple chemical reaction (Van Stockar et al., 1993; Gustafsson, 1991). What is measured by calorimetry is the sum of the heat exchanges related to the biological process and physico-chemical reactions. At constant pressure, such heat exchanges correspond to enthalpy variations. The continuous detection of the heat exchanged (released or uptaken) by a microbial culture allow the monitoring of the microbial growth and other microbe-sustained processes that affect the matrix of the culture and produce chemical and physical changes in the surrounding medium (Olz et al., 1993; Battley, 1987). It has been shown that the release of heat from microbial cultures can be used to on-line monitor growth and metabolism of cells (Yi et al., 2000; Vandenhove, 1998; Lamprecht, 2003; Wadsö & Galindo, 2009). For this reason, Calorimetry can be successfully applied also to spoilage processes and shelf-life determinations (Wadsö & Galindo, 2009; Riva et al., 2001; Alklint et al., 2005; von Stockar & van der Wieler, 1997).The great advantage of calorimetric measurements for biological studies is that the technique can be used to study any system undergoing changes, since it is not affected by physical or chemical peculiarities. The drawback lies just in this non-specificity, which makes this technique " blind" if not supported by other pieces of information about the system investigated. In the case of microbial cultures, the additional data concern plate counts, substrate concentration, pH changes, etc. (Boe & Lovrien, 1990; Menert et al., 2001). These parallel experiments are of great help to the interpretation of the calorimetric measurements.

1.3 CALORIMETRIC METHOD IN STUDIES OF BACTERIAL GROWTH

Isothermal calorimetry has been used in the investigations of different experimental studies of bacterial growth during the last 50 years. The first experiments, carried out with *Streptococcus faecalis*, showed that the rate of heat production was coupled with the rate of biomass growth (Forrest and Walker, 1963). Later studies concerned the growth of *Escherichia coli, Staphylococcus aureus* (Zaharia et al., 2013), *Lactobacillus helveticus* in different environmental conditions (Liu et al., 1999) etc..Some other studies were devoted to the thermodynamic and kinetic parameterization of the processes (Beezer, 2001; Rong et al., 2007; Maskow & Babel, 2003; Schäffer et al., 2004; Higuera-Guisset et al., 2005; Peitzsch et al., 2008). It was also proved that calorimetry can provide information about different

metabolic changes such as shift from one substrate/type of catabolism to another, occurrence of limitations and inhibitions (Yi et al., 2000; Winkelmann et al., 2004; Yao et al., 2008).
The elaboration of enthalpy data drawn from calorimetric measurements allows one to discover the formation of unknown or unexpected products (Maskow & Babel, 2003; Yao et al., 2008), or to study the synthesis of intermediate products of metabolism and their effect on bacterial growth (Wang et al., 2005). Calorimetric power-time curves could be used also as finger prints of a given microbial process, as the shape and the number of phases of these curves depend on the composition of the bacterial consortia (Vandenhove, 1998) and reflect complicated patterns of multi-stage growth processes.

1.3.1. Models to fit calorimetric traces related to microbial growth

The bacterial replication can be monitored in real time through the heat released that is recorded as heat flow versus time (Perry et al., 1979; Schiraldi, 1995). Taking into account that the heat flow, HF is the power recorded, it can related to the rate of the underlying process:

$$HF = \frac{dQ}{\mathrm{d}t} = \dot{Q} = \dot{\alpha} \times \Delta H$$

where $\dot{\alpha}$ and ΔH are the rate and the enthalpy drop of the process, respectively. Usually, the calorimetric trace, HF-vs-t, shows a neat exothermic peak just when the maximum growth rate is expected. The signal however reflects both growth, namely, the increase of the number of CFU, N, and their metabolism, which is active even when no cell duplication is taking place. This implies that the peak shaped signal is someway skewed and shouldered, being the sum of these two main contributions, namely,

$$\frac{dQ_{gr}}{\mathrm{d}t} = \frac{\mathrm{dN}}{\mathrm{d}t} \times q_{gr} \quad (1)$$

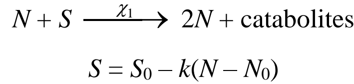
Related to the cell duplication and proportional to dN/dt , and

$$\frac{\mathrm{d}Q_{met}}{\mathrm{d}t} = \mathrm{N} \times \frac{\mathrm{d}\,q_{met}}{\mathrm{d}t} \quad (2)$$

Related to the metabolic activity and proportional to N, where q_{gr} and $\mathrm{d}q_{met}$ /dt stand for heat and heat flow rate, respectively, from a single cell and can be treated as constant quantities when an excess feeding substrate is present. The overall heat flow rate is therefore given by:

$$\mathrm{HF} = \dot{Q}_{tot} = \left(N \times \frac{dq_{met}}{dt} \right) + \left(\frac{\mathrm{d}N}{\mathrm{d}t} \times q_{gr} \right) \quad (3)$$

A growth model aims at the definition of a suitable function for N(t) which may be tested by fitting the calorimetric trace and looking for the best values of (dq_{met}/dt) and q_{gr}.
The model must take into account the overall conditions of the microbial culture, namely the starting value of N, the amount of the feeding substrate, the presence of inhibitors of the growth, or bactericidal compounds, etc. For this reason preliminary investigations are normally needed to assess the best (or the worst) conditions for the development of a given microbial culture. When the model implies several steps, one must take into account the rate of each step, normally with a differential equation. Eventually the model is defined by a system of differential equations that must be integrated to yield the corresponding function N(t).
The simplest model that can be suggested is (Model 1):



where N and S stand for the number of CFU mL-1, and substrate concentration, respectively. χ_1 is the respective kinetic constant of growth and k is related to χ_1 to account for the substrate consumption. The corresponding growth rate can be described with the expression:

$$\dot{N} = \frac{dN}{dt} = \chi_1 \times N \times S \qquad (4)$$

Integration of equation (4) gives the function *N*(*t*), namely,

$$N(t) = \frac{A\exp(Bt)}{1 + C\exp(Bt)} \qquad (5)$$

Where

$$A = \frac{N_0}{S_0}\left[S_0 + kN_0\right];\ B = \chi_1\left[S_0 + kN_0\right]\ and\ C = \frac{kN_0}{S_0}$$

(the subscript “0” stands for starting value)
The corresponding heat flow, Q_{gr}, is proportional to the growth rate, dN/dt,

$$Q_{gr} = \dot{N} \times Q_{gr} \qquad (6)$$

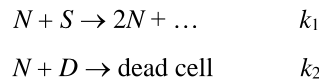
And

$$Q_{met} = N \times \dot{Q}_{met} \qquad (7)$$

The overall Q is

$$\dot{Q}_{tot} = \dot{N} \times Q_{gr} + N \times \dot{Q}_{met} \qquad (8)$$

This model can be made more complex to include the effect of some toxic compound, D, that kills the cells with a lethal dose, D, per single cell (Model 2).



where the k_i are kinetic constants.
With the starting (t = 0) parameters: N_0, RS = S_0/N_0, RD = D_0/N_0, K = k_2/k_1, and assuming a unit value for k1, one obtains the following system of differential equations:

$$\dot{N} = N \times S - K \times N \times D$$
$$\dot{S} = -N \times S \qquad (9)$$

$$\dot{D} = -K \times N \times D$$

with

$$\dot{N}_0 = R_s \times N_0^2 - K \times R_D \times N_0^2 = (R_s - K \times R_D \times N_0^2)$$
$$\dot{S}_0 = -R_s \times N_0^2$$
$$\dot{D}_0 = -K \times R_D \times N_0^2$$

The model implies that

$$\dot{N} + \dot{S} - D = 0 \qquad (10)$$

which, by integration from $t = 0$ to t, yields

$$N = (N_0 + S_0 - D_0) - (S - D) \qquad (11)$$

that fixes the maximum attainable value of N, namely,

$$N_{\max} = N_0 + S_0 - D_0 \qquad (12)$$

Both steps imply some heat exchange. The cell duplication is expected to be exothermic, while no expectation may be envisaged for the bactericidal step.

$$\dot{Q}_{dupl} = -q_{gr} \times \dot{S}$$
$$\dot{Q}_{death} = q_D \times \dot{D} \qquad (13)$$
$$\dot{Q}_{met} = \dot{q}_{met} \times N$$

This means that if the decrease rate of the substrate and the toxic compound are monitored, one can define suitable functions (fitting) for S(t) and D(t), which allow to draw N(t) through equation (11). Eventually one may attain an expectation for $\dot{Q}_{tot}$ to be compared with the experimental evidence collected with an isothermal calorimetry investigation. $\dot{q}_{met}$

From such a comparison the best values for $\dot{q}_{met}$, q_{gr}, and the kinetic constants can be assessed.

Combinations of these two models can be considered when a given culture may host two or more microbial species, each with its own growth parameters.

A variant that should be included in Model 2 concerns the possibility that the toxic compound may be the product of the metabolism. In such a case a self-limitation of the growth would occur. The modifications to insert concern the compound D. The model becomes

$$N + S \rightarrow 2N + D \quad k_1$$
$$N + D \rightarrow \text{dead cell} \quad k_2$$
$$\dot{N} = N \times S - K \times N \times D$$

$$\dot{S} = -N \times S \qquad (14)$$
$$\dot{D} = N \times D - K \times N \times D = N \times D \times (1 - K)$$

with

$$\dot{D}_0 = -N_0 \times D_0 \times (1 - K)$$

D_0 being naught or fixed at some fixed value.
In such cases D may correspond to some acid produced by the microbial metabolism (e.g., *Lactobacillus*). Once D reaches a maximum tolerable value (pH limit), the growth is arrested.

1.3.2. Advantages of IC

The study of microbial growth in solid matrices and in opaque liquid media has been previously described in many occasions (Malakar et al., 2000; Mitchell et al., 2004). The experimental methods used for the study of complex matrices measurements of OD, outplating, direct microscopy measurements, are quite cumbersome and not very informative about the peculiarities of growth. Currently more and more attention is drawn to use of calorimetry in studying and monitoring the metabolic activity of bacteria in opaque liquids, on the surfaces and in solids in wide range of conditions (Wadsö & Galindo, 2009; Braissant et al., 2010; Mitchell et al., 2004; Mihhalevski et al., 2011), It could be successfully applied in the studies of spoilage processes and shelf-life determination of products containing microbes (Riva et al., 2001; Wadsö & Galindo 2009), in fact in all cases where the possibilities of optical and other physical methods are limited due to the non-transparency of media (Wilson et al., 2002; Antwi et al., 2007).
Calorimetric power-time curves give a lot more information about the peculiarities of bacterial growth and its metabolism (Lago et al., 2011; Stulova et al., 2011), which hardly could be obtained using conventional methods - multiauxic growth, switch from one substrate to another etc. Calorimetry provides also an efficient alternative for the precise determination of the specific growth rates (Liu et al., 1999). Moreover, calorimetric method allows detect the concentrations of bacteria in the range of 10^4-10^5 cells, which could not be detected using traditional methods (Braissant et al., 2010).
It has to be emphasized that the power signal is quantitatively proportional to the number of viable organisms in the sample, which means that the technique may not erroneously include not living cells.
Despite the fact that power-time curves provide a lot of information on the growth it is very useful to collect some additional data using other methods in parallel (Chen et al., 2008).
Calorimetric method has definite advantages in comparison with other methods used in microbiology:

- calorimeter measures the heat produced by the growth of bacterial culture that depends not only on the biomass amount, but also on the amount of metabolized substrate and on the efficiency of carbon source utilization;
- calorimeter gives more information for the calculation of the energy balance and the efficiency of metabolism;
- the growth of bacterial culture could be continuously recorded with the calorimeter and without growth disturbance;
- calorimetric measurements provides a good repeatability of the growth curves;
- it is possible to analyze opaque and turbid samples, it means that the technique does not require optical transparency of samples and is invariant irrespective of their physical form;
- The instrument requires no specific sample treatment or preparation, as the samples are simply housed within an ampoule and monitored in situ (Gaisford et al., 2009; Vandenhove, 1998);
- The theory behind the technique is well developed, which facilitates interpretation of the data (Höhne et al., 2003).

1.3.3. The instrument: Isothermal Calorimeter "DAM"

The instrument used in this investigations was the "Calorimètre E. Calvet pour Microcalormétrie, DAM" (Setaram, Lyon, France) (Fig 1.1) equipped with 12 cm3 volume capacity stainless steel measurement compartment (Fig 1.2) and the injection system Microlab 500 (Hamilton Company, Reno, RV). Calibrations were performed using the Joule effect calibrator EJ2 (Setaram). The Heat Flux versus time raw signal was numerically integrated to obtain the overall specific enthalpy (with respect of grams or milliliters of total cells amount) ΔH (J g^{-1}) or (J ml^{-1}).

Figure1.1. Photography of the isothermal calorimeter DAM

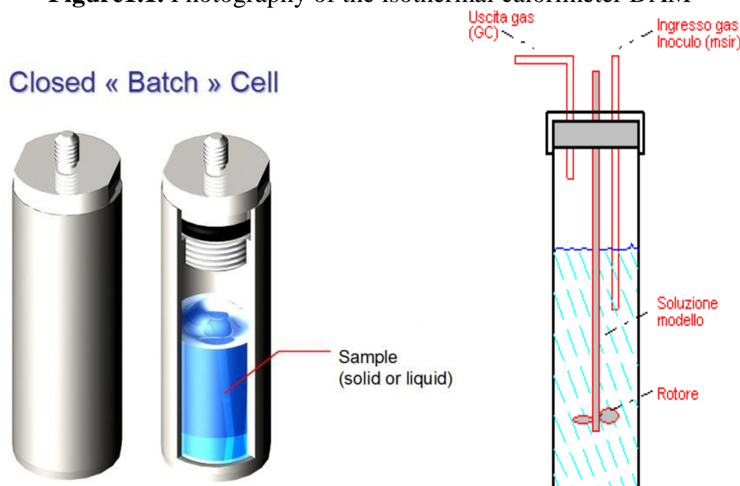


Figure 1.2. Calorimetric cells. *Right:* scheme of the injection system of a calorimetric cell.

DAM is instrument where the heat output or input associated with a given phenomenon is transferred between a reaction vessel and a surrounding thermostat jacket. The temperature of the jacket is kept constant and is to a good approximation unaffected by the heat exchange with the reaction cell. The jacket is therefore designed to function as a heat source/sink of virtually infinite heat capacity.
The heat transfer between the reaction vessel and the jacket can be monitored with high thermal conductivity thermopiles containing large numbers of identical thermocouple junctions regularly

arranged around the reaction vessel (the cell) and connecting its outside wall to the heat source/sink (the thermostat). The determination of the heat flow relies on the so-called Seebeck effect. An electric potential, known as thermoelectric force is observed when two wires of different metals **1** and **2** (Figure 1.3) are joined at both ends and these junctions are subjected to different temperatures, T1 and T2 (Figure 1.3.a). Several thermocouples can be associated, forming a thermopile (Figure 1.3.b).

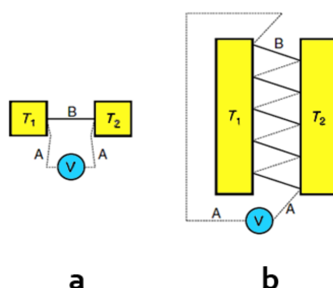


Figure 1.3. A thermocouple (a) and a thermopile (b) as devices for measuring a temperature difference or a heat flow. A and B are wires of different metals.

A heat flow calorimetry experiment is performed as follows: (*i*) initially the calorimetric cell and the jacket are in equilibrium and therefore $T1$=$T2$ (that are equal to the thermostat temperature) leading to a zero heat flow; (*ii*) when the process under investigation is initiated, $T2$ is changed due to the absorption or release of heat by the reaction under study. This small temperature difference generates a thermoelectric force that, when converted to heat using the calibration constant of the calorimeter (obtained by Joule effect) (see figure 1.4), can be associated with the enthalpy of the process under investigation.
The instantaneous thermal power generated, P = dQ/dt, is measured by means of a heat-flow sensor located between the sample and a heat sink. The time integration of the thermal power traces gives the total heat, Q, associated with a biological process.

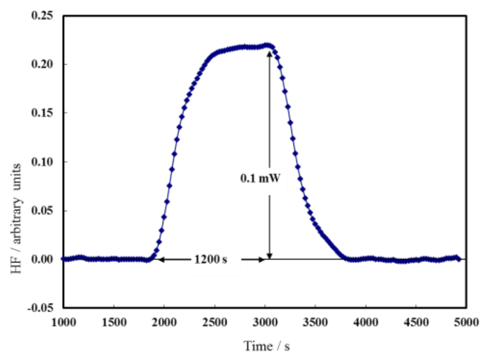


Figure 1.4. Example of a characteristic calibration curve.

1.3.4. Analysis of power-time curves – the output of calorimeter

The curves that are the output of calorimeter are called calorimetric thermo-grams or power-time curves. Power-time curve reflects the time derivative of a standard bacterial growth curve in batch cultures. Calorimetric power-time curves (see Figure 1.5) could be divided into three growth phases) essentially the same way as the bacterial growth curves, but with some reservations. Firstly goes the lag-phase, which determines the adjustment period during which bacterial cells adapt to the new environment and start to divide exponentially. The length of the lag-phase was determined as shown on Figure 1.6. The calculation procedure was practically the same as in Swinnen et al. (2004). However, it should be emphasized that in calorimetric experiments the length of the lag phase does not directly reflect is the physiology of the cell, but mainly depends on the sensitivity of the calorimeter used.
Sensitivity of DAM in registration of heat, in our conditions, is about 10 µW, which means that only a certain number of growing (metabolizing) bacteria can produce heat fluxes surpassing the sensitivity threshold of the instrument. It means that the lag-phase duration measured on the basis of calorimetric curves should be calculated taking into account the time when the heat produced by the growing bacterial population exceeds the level of the sensitivity of the instrument (Swinnen et al., 2004).
The second phase of the power-time curve after the lag-phase corresponds to the exponential growth phase during which bacteria grow with maximal growth rate, μ_{max}, in the experimental conditions.
The heat fluxes are proportional to the growth of cells, heat flows are equivalent to the growth rate values. The end of exponential phase of bacterial growth was defined in our data analysis by the peak of the power-time curves. According to the results obtained by *plate count*, the end of exponential

growth could be put at some time after the peak of calorimetric curve. In the ideal systems the results obtained by plate count can perfectly correspond to the integrated power-time curve (Figure 1.7).

The last phase of microbial growth is called deceleration phase and it's corresponding in our case to the area coming immediately after the peak of calorimetric curve.

All calorimetric experiments were performed in three parallels and obtained power-time curves were normalized per milliliter in case of bacterial growth in liquids and in grams in the of solid matrices. Averaged power-time curves of three parallel runs were used for the further analysis.

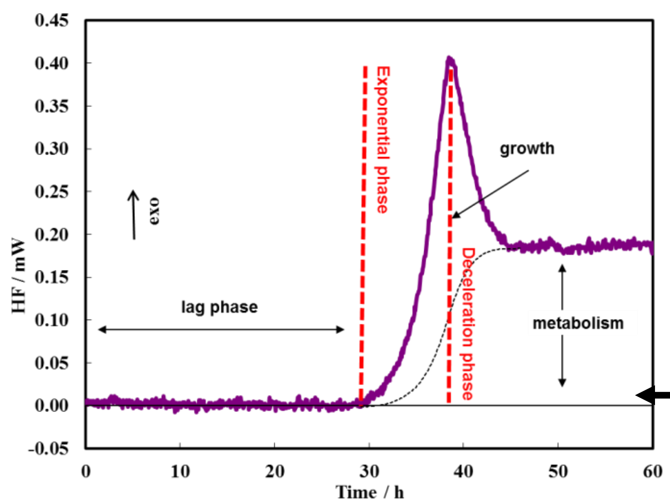


Figure 1.5. The explanation of the processing of calorimetric power-time curves; the division of calorimetric power-time curves into three phases: lag phase, exponential growth phase, and deceleration phase, and areas below the curve, corresponding to metabolism and growth.

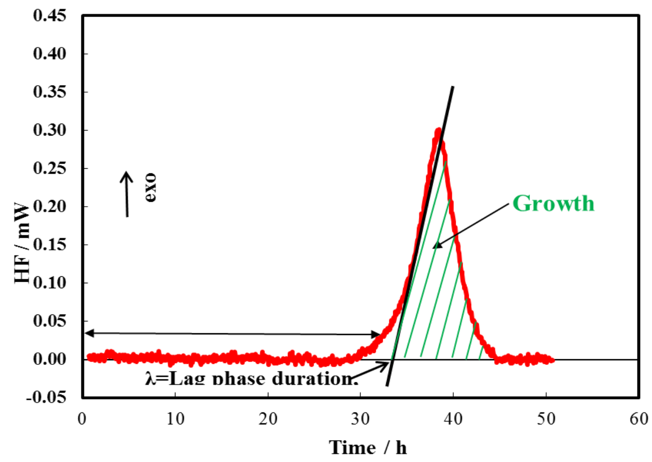


Figure 1.6. Determination of the maximum growth rate (μ_{max}, h^{-1}) and the lag phase duration (λ, h).

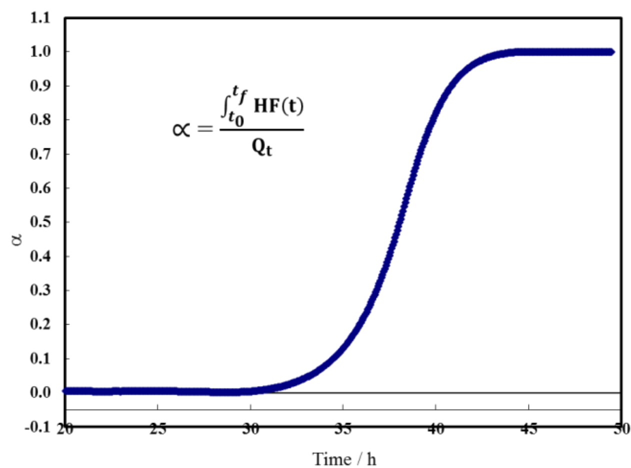


Figure 1.7. The integrated power-time curve: accumulated heat change in time.

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2. ISOTHERMAL CALORIMETRY INVESTIGATIONS OF MICROBIAL GROWTH IN ARSENIC CONTAMINATED MEDIA

2.1. STATE OF THE ART

2.1.1. Bacterial growth in soils

In soil, microorganisms are real bio-indicators of soil quality; they are able to perform an extremely wide range of chemical transformation including degradation of organic matter, disease suppression, disease, nutrient transformations and the overall metabolic activity.
Soil is a variable mixture of minerals, organic matter, and water, capable of supporting plant life on earth's surface. The organic portions of soil consist of plant biomass in various stages of decay.
Soil microorganisms include a large variety of microorganisms such as bacteria and fungi, for (Hattori, 1973), almost 80–90% of the microorganisms inhabiting soil are on solid surfaces, and there is a strong relationship between the microflora and the quality of the soil since microbes are involved in the decomposition of organic matter. Bacteria in soil usually carry out the greater fraction of soil biochemical process. This is especially true of anaerobic process, which fungi cannot mediate. The most important peculiarity of bacteria is their variable nutritional and metabolic activity. Soil bacteria degrade biomass, destroy xenobiotic contaminants and mediate oxidation/reduction process involving carbon, nitrogen, sulfur, iron, and manganese species. Various kinds of bacteria may require nutrients, such as amino acids or vitamin that they cannot produce by their own, but may be supplied other organisms, including other kinds of bacteria.
The microbial communities that grow first and most rapidly are those that degrade common food materials such as carbohydrates, proteins, and organic acids from lipids. Number of such bacteria increase rapidly when readily metabolized nutrients are added to soil, and their metabolic activity diminishes abruptly when the nutrient sources are removed.
The growth of some microbial species reaches some size limit is imposed by one or more limiting factors, such as:
Environmental factors: High temperature, drought and soil acidity as major causes of nodulation failure, affecting all stages of the symbiosis and limiting rhizobial growth and survival in soil. (Hungria & Vargas, 1999).
Effect of clay: the type and amount of clay present in soil can affect the soil matrix, and therefore, bacterial survival and activity.
Effect of oxygen: in aerobic conditions, for nitrogen fixing organism, such as *Azotobacter*, respiration and nitrogen fixation are alternative pathways of oxidation, the hydrogen of reduced substrates being transferred ultimately in the former case to oxygen and to nitrogen in the latter one.
Effect of nutrients: lack of carbon has been assumed to be the most common limiting factor for bacterial growth in soil, although there are reports of limitation by other nutrients, like nitrogen and phosphorus. At low growth rates most of the carbon is used for maintenance, while a minor fraction is available for growth and biomass production.

2.1.2. Microbial growth and heavy metals

Heavy metals present in soil are a serious threat to human and animal health. Neither are they neutral to plants (Belyaeva et al., 2005) or microorganisms (Wyszkkowska et al., 2007). They can have an inhibitory effect on the development of bacteria (Baros et al., 2007; Lugauskas et al., 2005). Heavy metals reduce biomass of microorganism and lower their soil activity (Wyszkowska et al., 2008; Min et al., 2005) and even they do not reduce their number, they depress their biodiversity (Moffett, 2003).
The mechanism of heavy metals affecting the environment in the soil-plant relation has not been completely clarified. On the one hand, soil bacteria immobilize heavy metals contributing to higher mobility of heavy metals, which is mainly due to metabolites they produce (Kuffner et al., 2008).
Microorganisms can mobilize metals through autotrophic (chemo-lithotrophic leaching) and heterotrophic leaching (chemo-organotrophic leaching), chelation by microbial metabolites, and

methylation, which can result in volatilization. Conversely, immobilization can result from sorption to cell components or exo-polymers, transport into cells and intracellular sequestration or precipitation as insoluble organic and inorganic compounds (Sayer & Gadd, 1997), sulphides or phosphates (Young & Makaskie, 1995; White & Gadd, 1996). In bioremediation, solubilization provides a route for removal from solid matrices such as soils, sediments, dumps and industrial waste.

2.1.3. Microbial growth and arsenic

Arsenic is a semi-metallic element, which may be found in a variety of form, viz. -3, 0, +3, +5 valences. The most common arsenic species observed in the environment are the trivalent form arsenite As(III) and pentavalent form arsenate As(V). Arsenate is often found co-precipitated with iron oxyhydroxide (FeOOH), which may be immobilized under acidic and moderately reducing conditions.
Under reducing conditions, arsenic is found as arsenite which can co-precipitate with metal sulfides (Niggemyer et al., 2001). In addition, numerous environmental factors also influence arsenic speciation in soil, such as pH, redox potential, the presence of other ions, organic matter content, soil texture, fungal or bacterial activities (Turpinen et al., 1999).
The toxicity of different forms of arsenic decrease in the order: arsine>inorganic arsenite>organic arsenite>inorganic arsenate>organic arsenate>free arsenic (Mandal & Suzuki, 2002). Toxicity depends on factors such as physical state, gas, solution, or powder particle size, cell adsorption rate, elimination rate, and the nature of chemical substituent in the toxic compound (Anderson & Cook, 2004).
Arsenic (As) is ubiquitous in soils and its concentrations range from 1 to 40 mg As kg^{-1} in uncontaminated soil, but can reach much higher levels in contaminated soils (Mandal & Suzuki, 2002). Arsenic mainly occurs in two inorganic forms, arsenite As(III) and arsenate As(V), both of which are highly toxic. Either forms are found in the environment, although As(III) is generally found in reducing environments, while As(V) predominates in well-oxidized conditions (Ackermann et al., 2008). The metabolism of microorganism influences the biogeo-chemical cycle of As, affecting both its speciation and toxicity. The tendency for As(III) to mobilize into the aqueous phase results from the differences in the sediment sorption characteristics of As(V) and As(III) (Smedley & Kinniburgh, 2002). Bacteria develop different As-resistance mechanisms that can be divided into two basic categories consisting either of redox reactions that conserve the energy gained for cell growth or detoxification reactions through the ars operon genes (Oremland et al., 2005; Silver & Phung, 2005).
The detoxification pathway occurs either aerobically or anaerobically and is not necessarily governed by prevailing redox conditions (Jackson et al., 2001). Arsenate respiration and detoxification are microbial processes that both contribute to As mobilization in ground water and soil (Oremland et al., 2005). However, the available information on arsenic behavior in agricultural soils is limited compared to ground water.
Accordingly, it is obvious that arsenic distribution and toxicology in soils has become a serious issue.
Calorimetry has been extensively used in studying the interaction between heavy metals and microorganism (Barja et al., 1997). Being a universal, integral, nondestructive, and highly sensitive tool for many biological investigations, the isothermal calorimetric technique directly determines the biological activity of a living system and provides a continuous measurement of heat production, thereby giving both extensive qualitative and quantitative information (Critter et al., 2004; Yao et al., 2005).

2.1.4. Calorimetric method in studies of bacterial growth in soils

Non specific analytical techniques like calorimetry have an advantage in a broad range of applications. This method has proven to be a suitable technique for measuring the microbial activity in complex systems, and is able to monitor aerobic as well as anaerobic metabolic processes (Barja et al., 1997). Thus, for different kinds of living systems, the measurement of thermal effects was applied in soil,

sludge, and waste water systems (Sparling, 1983). Isothermal calorimetry applied to microbial processes when an external agent is added was found to be a useful microbiological technique, with a promising future. The thermal effect involving glucose degradation provided information on the microbial activity of the soil microorganisms that metabolize glucose (Airoldi & Critter, 1996; Wadsö, 1997).

The classical microbial activity determination in soil directly measures carbon dioxide evolution (respirometry), biomass (by the amount of carbon or nitrogen mineralized), and plating count of microorganisms growth (Anderson & Domsch, 1978; Parkinson & Paul, 1982).

Each specific method for microbial activity measurement has its limitations. Microscopic techniques involve direct counting of only a minute part of the soil microorganisms growing in plates. These require an expert researcher to distinguish between living and dead cells.

In addition, the quantity of a particular cell component can vary considerably with growth conditions (Anderson & Domsch, 1978). On the other hand, enzymatic activities require optically clear solutions, measured using spectrophotometric methods (Bandick & Dick, 1999). This problem can be overcome by microcalorimetry, which continuously quantifies the microbial activity in real time, with the incubation time being the same in the experiments and in actual soil conditions. This procedure is quicker than measuring separate component groups of microorganisms and feasible also in non transparent systems (Barros et al., 1999). Therefore, the calorimetric method has been proven to be very sensitive toward changes in the microbial biomass that cannot be detected with conventional methods (Vandenhove et al., 1991).

2.2. AIM OF THE STUDY

This PhD thesis research project aims to develop a practical calorimetric protocol for the studies of bacterial growth in liquid cultures and solid cultures focusing the attention on the study of two different strains aerobic and anaerobic.
Specifically, the aims were as follows:

- To develop the analytical method for the studies of bacterial growth using Isothermal calorimetry with other analytical methods on the basis of the growth of *Bacillus* sp. 3.2 and *Achromobacter* sp. As5-13 in liquid cultures as a model that can be realised in solid cultures (soils);
- To compare the details of growth in the presence and the absence of arsenic and investigate the bacterial growth in different conditions;
- To evaluate the use of calorimetric method for the study of the toxic effect of arsenate As(III) and arsenite As(V) on the microbial growth;
- To evaluate the effect of different kind of cultures media on the toxic effect of arsenic on the growth of *Bacillus* sp. 3.2.

2.3. MATERIALS AND METHODS

2.3.1. Bacterial strains studied

2.3.1.1. *Achromobacter* sp. As 5-13

Achromobacter is a genus belonging to the family Alcaligenaceae in the order Burkholderiales. The cells are gram negative straight rods and they are strictly aerobic and are found in water (Jianghan et al., 2013) and soils (Buckova et al., 2010). Particularly the strain As5-13 used in this study was isolated from the rhizosphere of the wild *Cirsium arvense* (L.) plant from an arsenic polluted soil in Italy (Cavalca et al., 2010). It was provided by Dr. Vicentina Andreoni from Laboratory of Microbiology, University of Milan. The main characteristics of the strain are reported in Table 2.1.

Table 2.1. Arsenic-resistance genes and plant growth promotion (PGP) characteristics.

PCR product		
ArsC[a]	ArsB[b]	
-	+	
Phenotypic traits[c]		
ACC-deaminase activity	IAA production	Siderophore production
-	-	-

a , amplified with primers P52F/P323R (Bachate et al., 2009)
b , amplified with primers darsB1F/darsB1R (Achour et al, 2007)
c, from Cavalca et al. 2010.

As(V) resistant isolates enriched on BBMG with 15 mM arsenate, it's As(V) and As(III) resistance levels.
The resistance to As(V) and As(III) was evaluated by an increase in OD620 nm (from twofold to fivefold) of the inoculum. Collected data show that this strain has 200 and 10 mM resistance level towards As(V) and As(III), respectively.

2.3.1.2. *Bacillus* sp. 3.2

Bacillus is a genus of Gram-positive, rod-shaped bacteria and a member of the phylum Firmicutes. The metabolic network of *Bacillus* sp. can function in aerobic and anaerobic conditions, and test positive for the enzyme catalase. Ubiquitous in nature, Bacillus includes both free-living and pathogenic species. Under stressful environmental conditions, the cells produce oval endospores that can stay dormant for extended periods. *Bacillus* sp. 3.2 is arsenate reductase isolated from a soil containing pyrite cinders (Corsini et al., 2010), grow in TSA in 24. It was provided by Pr. Vicentina Andreoni from Laboratory of Microbiology, University of Milan.

2.3.2. Inoculum preparation

2.3.2.1. *Achromobacter* sp. As 5-13

Frozen storage cultures of *Achromobacter* sp. As 5-13 were thawed and pre-grown on TSA 1X plates for 24 h at 30°C. One colony from a pre-grown plate was used as an inoculum for a 10 mL culture in Tris Mineral Medium amended with 0.6% gluconate (TMMG) at 30°C. 1% of bacterial suspension grown overnight was used as inoculum for the successive 10 mL TMMG -culture and grown 24 h which allowed the *Achromobacter* sp. As 5-13 bacteria to reach the middle of the exponential growth

phase. Bacterial count was determined by plating on TSA 1X and incubating for 2 days at 30°C, and average number of bacteria in the mid-exponential culture was determined $(4.15\pm0.15)*10^{8}$ CFU $mL^{-1.}$

2.3.2.2. *Bacillus* sp. 3.2

A frozen cultures of *Bacillus* sp. 3.2 was thaw and pre-grown TSA 1X plates for 24 h at 30°C. One colony from a pre-grown plate was used as an inoculum for a 10 mL culture in Tris Mineral Medium amended with 0.6% gluconate (TMMG) at 30°C. 1% of bacterial suspension grown overnight was used as inoculum for the successive 10 mL TMMG and TSB culture and grown 24 h, the procedure was repeated one more time and the obtained bacterial suspension was further used for inoculation. Bacterial count was determined by plating on TSA 1X and incubating for 2 days at 30°C. The final average number of bacteria in the inoculum was $(1.15\pm0.19)*10^{9}$ CFU mL^{-1}.

2.3.3. Growth experiments

2.3.3.1. Medium composition for *Achromobacter* sp. As 5-13

TMMG (Tris Mineral Medium (TMM) supplemented with 0.6% (w/v) gluconate growth medium of the following composition was used (Table 2.2).
Solutions were prepared with deionized water and the pH was adjusted to 7.0 and then sterilized by autoclaving at 121 °C for 15 min.
Oligomineral solution was filtered with syringe filter 0.22 µm.

Table 2.2. The composition of the culture media TMMG.

Component	Amount
Tris HCl	1.39 g L^{-1}
NaCl	4.68 g L^{-1}
KCl	1.49 g L^{-1}
NH_4Cl	1.07 g L^{-1}
Na_2SO_4	0.43 g L^{-1}
$MgCl_2$ $6H_2O$	0.2 g L^{-1}
$CaCl_2$ $2H_2O$	30 mg L^{-1}
Na_2HPO_4	40 mg C
Fe(III)NH_4 citrate	0.048 %
Oligominerals	1 ml $^{-1}$
Component (oligominerals)	**Amount**
HCl (25 %)	10 mL
$FeCl_2$ $4H_2O$	1.5 g
CoCl $6H_2O$	0.19 g
$MnCl_2$ $4H_2O$	0.1 g
$ZnCl_2$	0.07 g
H_3BO_3	0.062 g
Na_2MoO_2 $2H_2O$	0.036 g
$NiCl_2$ 6 H_2O	0.024 g
$CuCl_2$ $2H_2O$	0.017 g

2.3.3.2. Medium composition for *Bacillus* sp. 3.2

For this strain we used two kind of medium TMMG previously mentioned and TSB (Tryptic Soy Broth) with the following composition (Table 2.3):
Solutions were prepared with deionized water and the pH was adjusted to 7.3± 0.2 at 25°C and then sterilized by autoclaving at 121 °C for 15 min.

Table 2.3. The composition for the culture media TSB.

Component	Amount
Enzymatic Digest of Casein	17.0 g L^{-1}
Enzymatic Digest of Soybean Meal	3.0 g L^{-1}
Sodium chloride	5.0 g L^{-1}
Dipotassium phosphate	2.5 g L^{-1}
Dextrose	2.5 g L^{-1}

Principles of the Procedure:
Enzymatic Digest of Casein and Enzymatic Digest of Soybean Meal are nitrogen sources in TSB. Dextrose is the carbon energy source that facilitates organism growth. Sodium chloride maintains osmotic balance; Dipotassium Phosphate is a buffering agent.

2.3.3.3. Chemicals (arsenic)

All chemicals were reagent grade. The stock solutions of As(III) and As(V) were prepared from AsNaO2 (Fluka) and Na2HAsO4.7H2O (Fluka), respectively. All solutions were prepared with deionized water and were syringe filter sterilized (0.2 mm, Millipore).

2.3.4. Isothermal Calorimetry (IC)

The growth of the microorganisms was monitored by means of IC. The instrument used was the "Calorimètre E. Calvet pour Microcalormétrie, DAM" (Setaram, Lyon, France) equipped with 12 cm^3 volume capacity stainless steel measurement compartment and the injection system Microlab 500 (Hamilton Company, Reno, RV). Calibrations were performed using the Joule effect calibrator EJ2 (Setaram). 5 ml of *Achromobacter* sp. As5-13 and *Bacillus* sp. 3.2 were loaded in the measurement compartment. Measurements where performed at 30 °C.
The Heat Flux versus time row signal was numerically integrated to obtain the overall specific enthalpy (with respect of grams of total cells amount) ΔH/J g. Three replicates were performed for each experiment. The ΔH errors were minor to 10%.

2.4. RESULTS AND DISCUSSIONS

2.4.1. The metabolic activity of *Bacillus* sp. 3.2 in arsenic contaminated media

Figure 2.1 reports a typical calorimetric trace (exothermic) related to the growth and metabolism of *Bacillus* 3.2 sp. in TMMG medium.

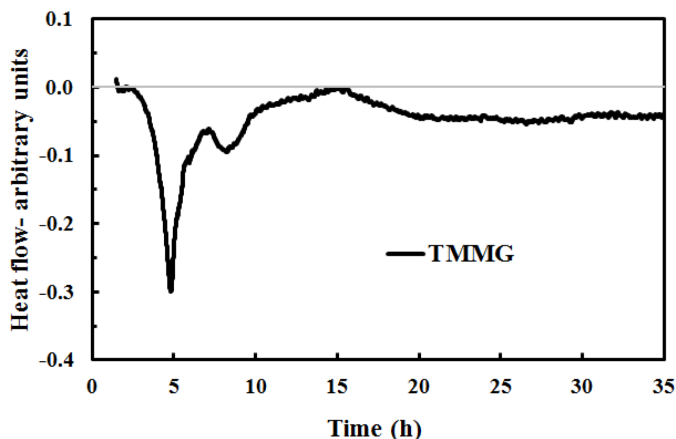


Figure 2.1. Power–time curves of the metabolic activity of *Bacillus* sp. 3.2 in TMMG at 30°C.

Figure 2.2 reports the plate count data performed in the traditional way (see Materials and Methods). The relevant trend shows that the microbial growth attains a plateau after 15-20 hours. This time lapse coincides with the end of the main calorimetric peak (see Figure 2.1). As mentioned above, this heat flux peak mainly reflects the rate of the growth process.

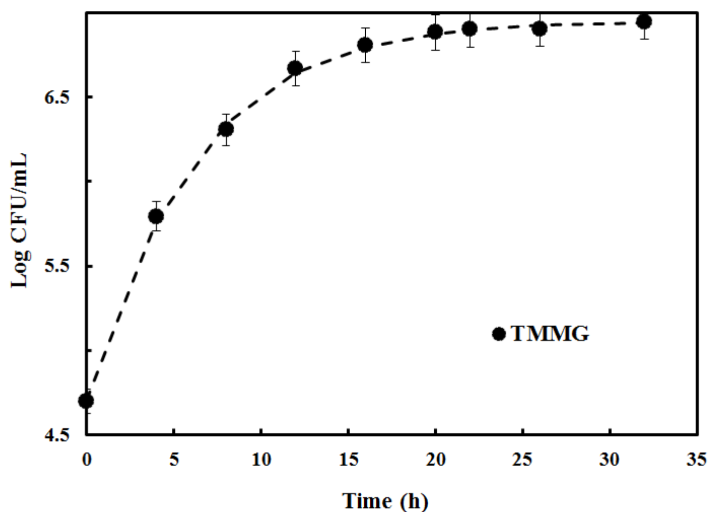


Figure 2.2. Microbial growth of *Bacillus* sp. 3.2 in TMMG.

This peak partially includes the contribution coming from the basal metabolism of the microbial

culture. Furthermore the signal actually is biphasic, which can related to some concomitant processes of various kinds. The details of such occurrence are out of the scope of the investigation which mainly aims at showing the typical calorimetric record collected from a microbial culture in the presence of accidental contaminants and extra effects. The signal tail that follows the growth peak reflects the metabolic activity of the viable cells.
Figure 2.3 allows the direct match between the calorimetric traces related to the microbial growth in the presence of As(III). Differences with respect to the record of the Arsenic free culture are not significant, safe for a slight delay in the growth onset. The overall enthalpy drop remains practically unchanged (see the values reported in Table 2.4).

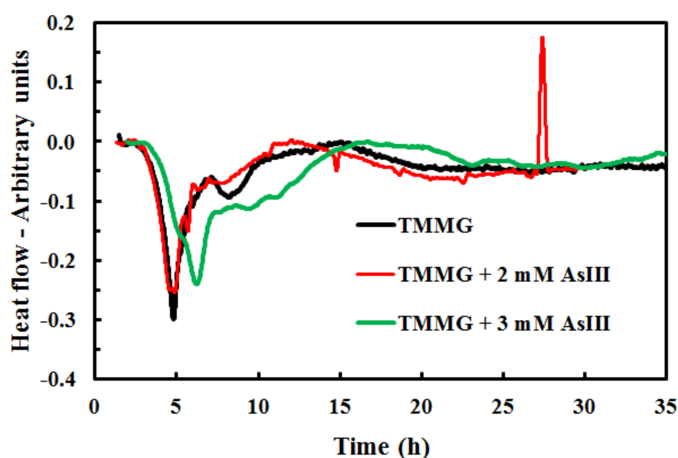


Figure 2.3. Power–time curves of the metabolic activity of *Bacillus* sp. 3.2 in TMMG in the presence of 2 and 3 mM of As(III) at 30°C.

Table 2.4. Enthalpy (ΔH) from power–time curves.

Culture media	**As(III) Concentration mM**	**As(V) Concentration mM**	**ΔH / mJ.mL^{-1} (err +/- 10%)**
TMMG	0	0	270
	1	0	270
	2	0	270
	3	0	260
	0	1	270
	0	2	250
	0	3	180
TSB	0	0	1100
	1	0	1095
	2	0	1090
	3	0	1090
	0	1	1080
	0	3	1000

These conclusions are in line with the results of traditional plate count investigations (see Figure 2.4).

Figure 2.5, related to microbial cultures in the presence of As(V) depicts a different situation. The main growth peak is substantially delayed and for 3 mmolar concentration of As(V) the thermal effect of the cell metabolism (tail signal) is drastically reduced.

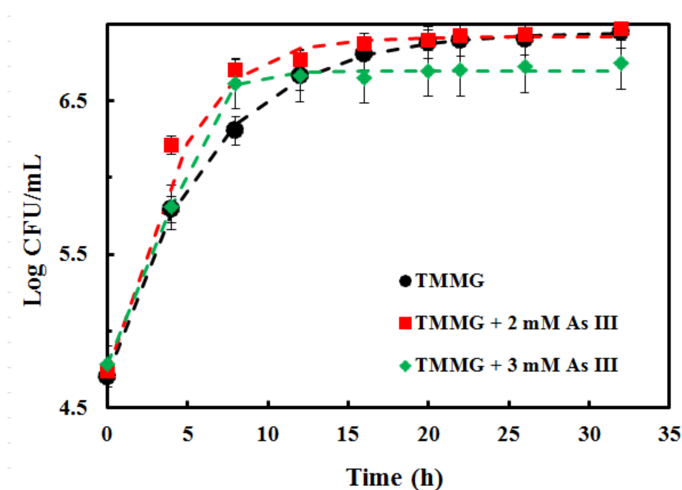


Figure 2.4. Microbial growth of *Bacillus* sp. 3.2 in TMMG, in the presence of 1, 2 and 3 mM of As(III).

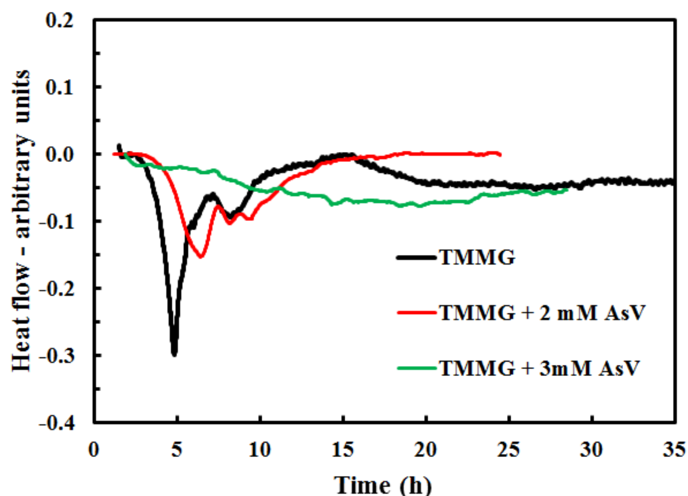


Figure 2.5. Power–time curves of the metabolic activity of *Bacillus* sp. 3.2 in TMMG in the presence of 2 and 3 mM of As(V) at 30°C.

Similar conclusions can be drawn from the results of classical plate count investigations (see Figure 2.6).

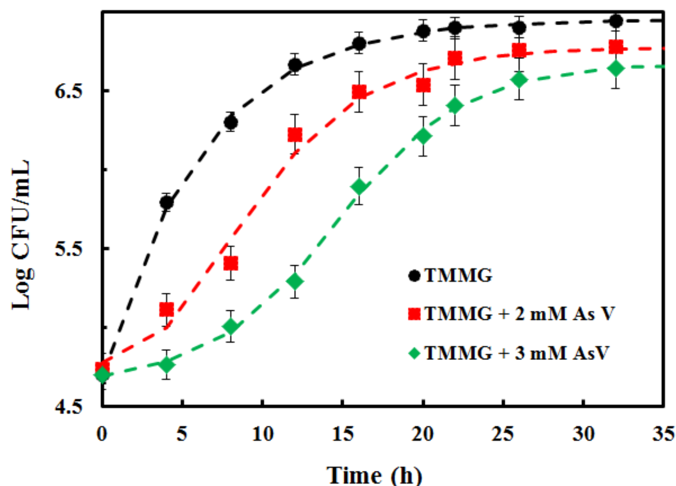


Figure 2.6. Microbial growth of *Bacillus* sp. 3.2 in TMMG, in the presence of 2 and 3 mM of As(V).

As a conclusion, one may say that in a nutritionally poor medium like TMMG the microorganism is able to survive and grow in the presence of As(III), while it is strongly inhibited in the presence of As(V) which seems rather toxic, as revealed by the growth delay and, above all, the severely reduced metabolic activity.
In order to check whether such a behavior may be referred to as a typical one, or is related to the specific poor medium, the calorimetric experiments were repeated in a rich medium.
Figure 2.7 shows the calorimetric records collected form microbial culture in TSB medium, with and without As(III). In either case the signal is much larger than those recorded from cultures on TMMG (see the relevant enthalpy values in Table 2.5). The main exothermic peak is practically identical in either case, safe for a slight onset delay. It is precede by a small peak which is delayed in the trace relevant to the culture containing As(III) 3 mM.
The end point of the main signal occurs at about 20 hours.

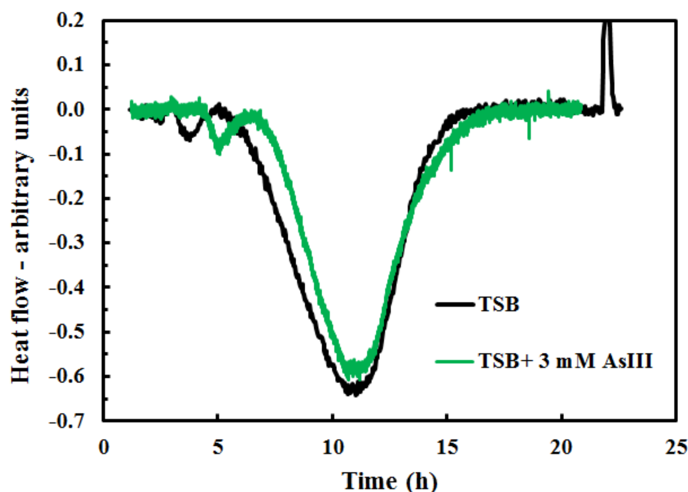


Figure 2.7. Power–time curves of the metabolic activity of *Bacillus* sp. 3.2 in TSB, in the presence of 3 mM of As(III) at 30°C.

Once again, these data are in line with those of plate count investigations (see Figure 2.8).

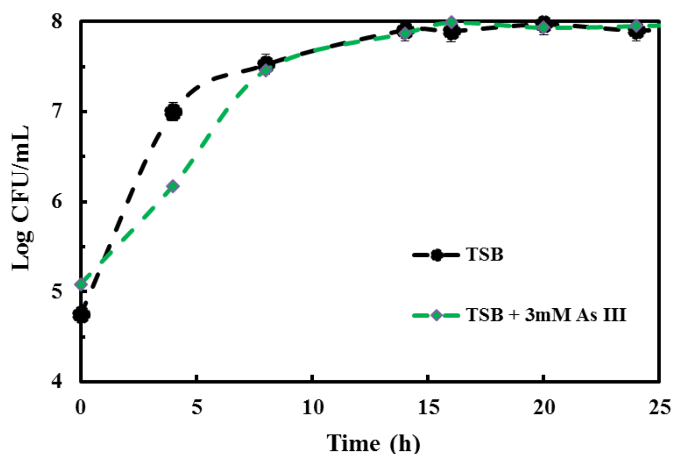


Figure 2.8. Microbial growth of *Bacillus* sp. 3.2 in TSB, in the presence of 3 mM of As(III).

It is therefore established that, even in a nutritionally rich medium, As(III) does not substantially affect the microbial growth and metabolism (see the relevant enthalpy values in Table 2.5).
Microbial cultures in the same medium but in the presence of As(V) behave differently (see Figure 2.9)

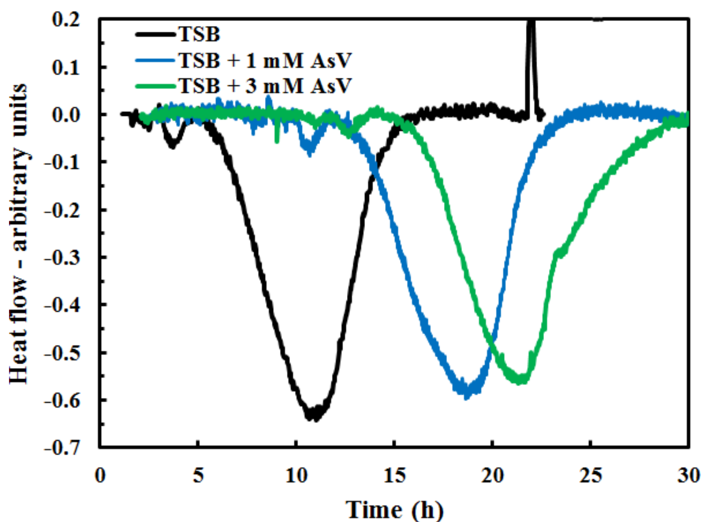
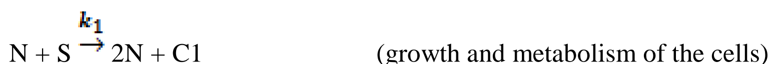


Figure 2.9. Power–time curves of the metabolic activity of *Bacillus* sp. 3.2 in TSB, in the presence of 1 and 3 mM of As(V) at 30°C.

When compared with the trace relevant to the As free culture, both As(V) 1 mM and 3 mM cultures show a substantial delay in either the main or the smaller peak. Nonetheless the relevant enthalpies are close to one another (which were not the case of cultures in TMMG). This reflects the fact that the increase of the microbial population, in spite of the delay produced by the presence of As(V), is practically unaltered.
One may therefore conclude that As(V) is indeed a toxic agent – if compared with As(III) - but its effects are significantly conditioned by the environment factors. In facts, when these conditions are favorable, the microbes are able to grow following the model 1 mentioned in chapter 1.
Such a behavior is indeed rather peculiar, as As(III) is commonly reported as the more toxic form.
The information drawn from calorimetric data is based on a suitable kinetic model for the growth process, so as to fit at the best the calorimetric signal. Tested models were those reported in chapter 1.
The model that gave the best fit of the calorimetric trace can be summarized as follows:


$N + S \xrightarrow{k_1} 2N + C1$ (growth and metabolism of the cells)

$N + As(V) \xrightarrow{k_2} As(V) + C2$ (toxic action of As(V))

where S, C1 and C2 stand for substrate and catabolites, while k1 and k2 are the relevant kinetic constants. The model requires the solution of a system of differential equations, under the condition,

$S = S0 – k1(N – N0)$

Where the subscript “0” stands for initial value. The overall growth rate, k, is a function of the [As(V)/N] ratio and therefore changes in the course of the growth process. The best fit trend is shown in Figure 2.10.

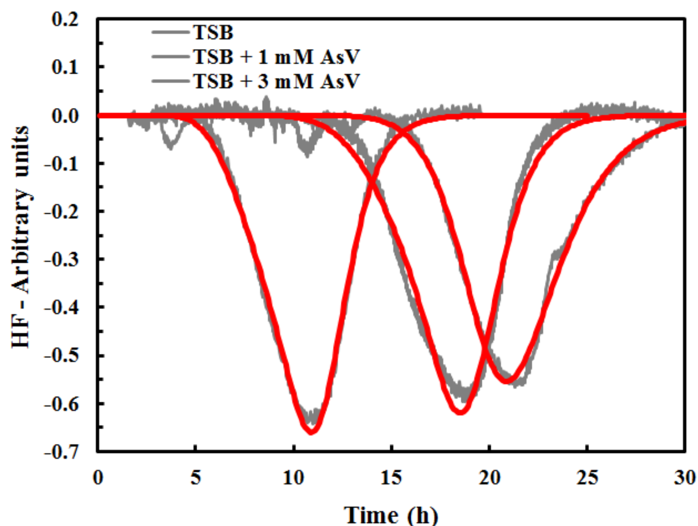


Figure 2.10. Fitting curves by the equation of Model 1 (Chapter1).

The very satisfactory fit supports the reliability of the selected model, that implies the invariant concentration of As(V), which is not consumed in the bactericidal activity. This invariance was verified in separate investigations (see materials and methods).

2.4.2. The metabolic activity of *Achromobacter* sp. As *5-13* in arsenic contaminated media

Figure 2.11 shows the exothermic peak recorded from a culture of *Achromobacter* sp. As 5-13 in TMMG medium. The growth trend is referred to the secondary Y axis. The match of the two traces indicates that the calorimetric signal becomes detectable only when a rather high threshold value of the microbial population has been attained. This reflects an energetically poor metabolic activity.
The calorimetric trace remains above the base line even when the microbial population does no longer increase. This reflects an independent metabolic activity that does not implies a further cell duplication.

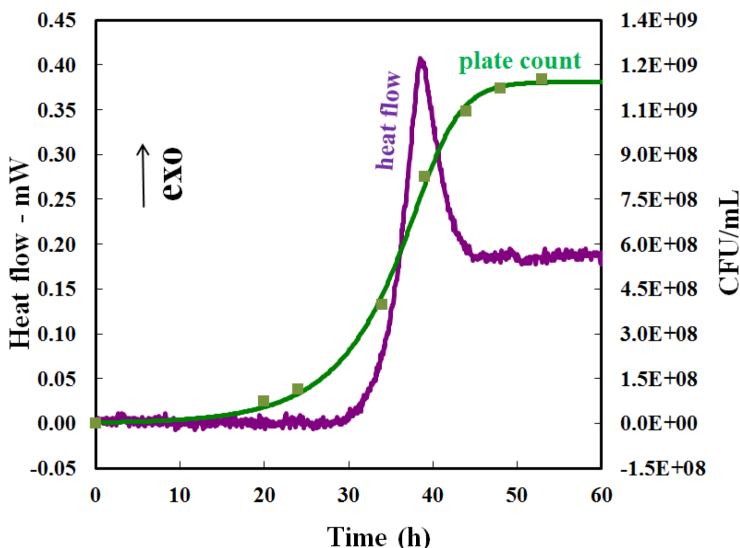


Figure 2.11. Heat flow trace (left vertical axis) and plate count (right vertical axis) of *Achromobacter* sp. As 5-13 in TMMG

Figure 2.12 shows the match of the calorimetric traces obtained from microbial cultures in a TMMG medium in the presence of As(III). Both cell duplication and residual metabolic after the duplication process are severely depressed. The reduced growth was also confirmed by plate count investigations (Figure 2.13).

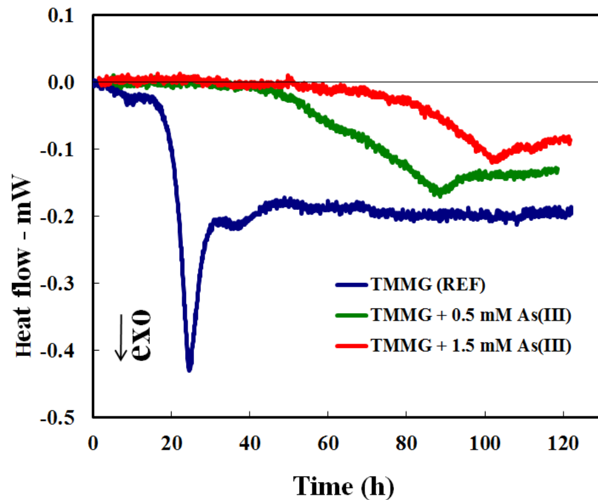


Figure 2.12. Calorimetric power-time curves of *Achromobacter* sp. As5-13 at different concentrations of As(III).

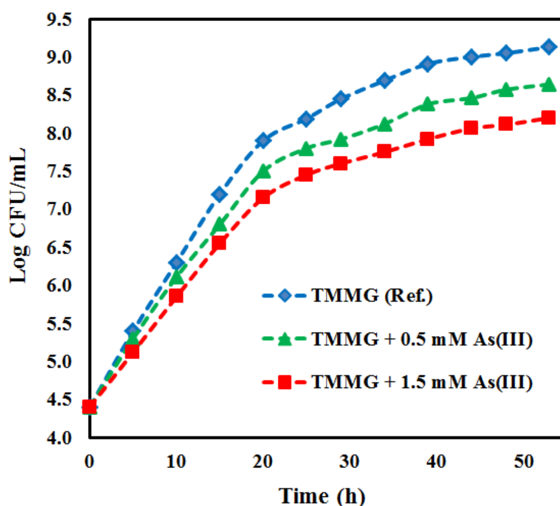


Figure 2.13. Microbial growth of *Achromobacter* sp. As5-13 at different concentrations of As(III).

In this case chemical analyses showed that the concentration of As(III) decreases (Figure 2.14 and 2.15) being oxidized to As(V).

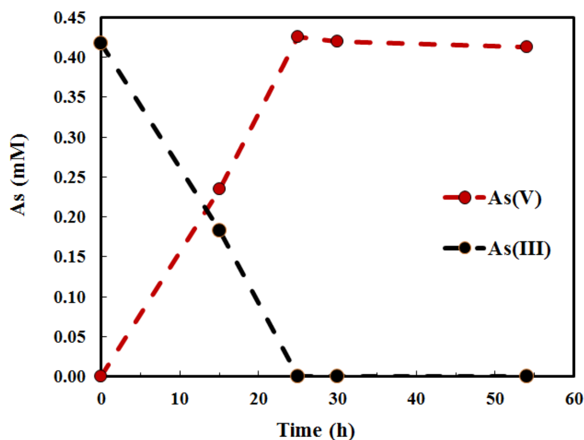


Figure 2.14. Oxidation of As(III) 0.5 mM to As(V) by *Achromobacter* sp. As5-13.

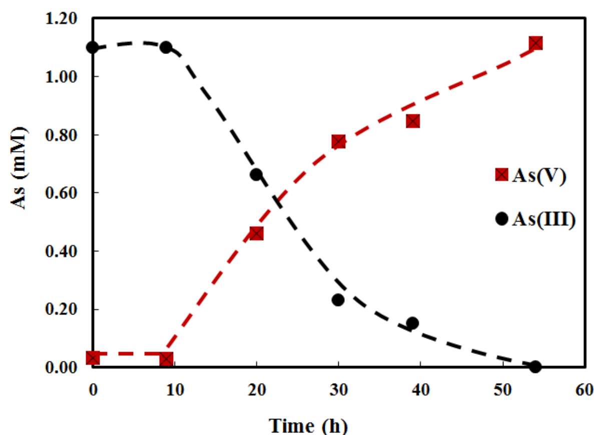


Figure 2.15. Oxidation of As(III) 1.5 mM to As(V) by *Achromobacter* sp. As5-13.

The calorimetric data show that, in spite of the presence of As(III), the growth of the microbes still takes place, although to a less extent with respect to As free cultures.
One has therefore to confirm some toxic action of As(III) on both growth and metabolism of the microbes, while, as mentioned above, As(V) would simply delay the onset of the growth process without affecting its overall energetics (the shape and the size of the exothermic peak remains unaltered).

2.5. CONCLUSIONS

The toxicity of As (either III or V) in soils may not be referred to as a general effect, since it depends on the specific microbial species, the relevant growth mechanism and catabolism of As, and the nutritional characteristics of the culture medium. One has therefore perform separate investigations for each single case considered.
Calorimetric data are to be combined with those of traditional experimental approaches to suggest suitable growth mechanisms that can account for pollutant-related effects either on the increase of the cell population or their metabolic activity.
The present results show that the calorimetric signals recorded from the culture of these microorganisms are rather weak and suggest the use of a high sensitivity equipment in further investigations.
Details of the present investigations will be reported in a ready to submit paper.

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3. EFFECT OF CUT ON MICROBIAL GROWTH OF CARROTS BY ISOTHERMAL CALORIMETRY

3.1. STATE OF THE ART

3.1.1. Carrots and derived products

Carrot (*Daucus carota*) is one of the most commonly consumed vegetables and represents one of the primary sources of carotenoids and vitamins A and B in the contemporary human diet (Bao & Chang, 1994). Raw and processed carrots are used in a wide variety of food products, including dehydrated soup mixes, chips, carrot juice, carrot nut muffins, oils and skin-care products. Among these products, carrot chips are quite popular and are available at practically all the leading food stores of the western world. The other use of minimally processed carrots is in carrot juice, which is becoming popular due to its high vitamin A contents. The vegetable juices available in the stores have a portion of carrot juice as a major constituent. Carrot or carrot juice frequent consumption has been associated to therapeutic effects in the treatment of chronic illnesses, including some types of cancer and cardiovascular diseases (Byers & Perry, 1992; Ruxton et al., 2006). Raw carrots are excellent or good source of β-carotene, vitamin B-complex, C, D and several minerals, in absorbable forms (USDA, 2011; Dattatraya, 2004).
The principal components of carrots are reported in the table 3.1.

Table 3.1. Composition of carrot (USDA, 2011).

Component	Amount
Water (%)	88
Energy (Kcal)	43
Protein (g)	1.0
Fat (g)	0.2
Carbohydrate (g)	10.1
Total fiber (g)	1.0

3.1.2. Quality attributes of fresh carrots during processing

Carrots as the majority of fresh fruits and vegetables are maintained as living tissues (respiring) until they are consumed (Aked, 2000). Carrot as a food product, as mentioned above, is characterized by a high overall nutritional value and by displaying some interesting functional features e.g., they are rich in antioxidant, fibre and vitamins and low in fat (Singh et al., 2000).
Consequently, many studies have investigated ways to process and extend the shelf life of this food while maintaining the beneficial quality. In the case of fresh consumption, slowing down senescence is a key factor to provide longer shelf life, this can be achieved by slowing respiration rates, and product deterioration (Aked, 2000). Slowing down respiration rates can be reached by cooling or using packaging such as modified atmosphere (MA) or controlled atmosphere (CA) to control quality deterioration (Toivonen & DeEll, 2002).
Convenient ready-to-use vegetables are in demand not only for the consumers but also by food service industries (Ahvenainen, 1996). Minimal processing technologies have been developed to provide fresh cut products with similar characteristics to fresh but in a ready-to-eat format. This commodity generally undergoes several steps such as trimming, peeling, washing and disinfection. However, these steps promote wounding responses, which accelerate deterioration and limit of shelf life (Aked, 2000). As expected, all processing implies the loss of freshness at some extent. In the case of fresh cut products, cell disruption influences the off-flavours appearance and browning, which occur due to reactions between cell constituents, in particular those that are catalysed by phenolase released from the tissue (Singh, 1994). Controlling this type of reactions requires a deeper understanding of the tissue structure and components to optimize the final quality.

In the case of fruits or vegetables, the cells biochemistry greatly determines chemical, physiological and biological properties of the tissue. For an example, see the role of pectin in cell walls of processed fruits and vegetables in a board review by Sila et al. (2009). The plant primary cell wall is mainly composed of pectin, cellulose and hemicelluloses. Variations in the composition will affect its functionality and the quality attributes of the product such as organoleptic perception, physiological responses to the environment and final quality.
During senescence, storage and processing, fruits and vegetables will present changes in colour, flavour, texture and nutritional value. The following table summarizes the major quality attributes of foods during processing and storage (Table 3.2)

Table 3.2. Quality attributes and undesirable changes during storage and processing (Singh & Anderson, 2004).

Quality attributes	Undesirable changes
Color	Off-colors (darkening, bleaching, browning)
Flavor	Off-flavors (hydrolytic, oxidative rancidity, “caramellization”)
Texture	Loss of solubility, water holding capacity, toughening or softening
Nutritive value	Vitamins, minerals, proteins or lipid degradation

3.1.3. Respiration

Plant tissues continue to respire after harvest. Over time they senesce and eventually die. Aerobic respiration results in oxygen and glucose consumption, production carbon dioxide, water and heat (Aked, 2000). It has been observed that products with greater respiration rates (oxygen consumption and carbon dioxide release measured in a certain time) typically have shorter storage lives, as excessive respiration causes metabolic collapse, breakage of cell membranes and cellular leakage (Aked, 2000) Environmental variations significantly influence rates of respiration. Subjected to the sensitivity to chilling injury, low uniform temperatures and air circulation are recommended to reduce respiration rate, heat liberation and condensation (Hardenburg et al,. 1986). The gas consumption surrounding the product will affect the physiology of the produce, taking into consideration packaging, film permeability, storage temperature and the level of wounding (Klaiber et al,. 2005).
Studies done on minimally processed carrots have shown that carrots can be stored as shredded packs showing gas equilibrium after 3-4 days at 4 °C due to respiration. Slicing carrots exhibited increase in respiration rates compared with intact carrots as expected (Barry-Ryan & O’Beirne, 1998), this was probably due to a wound response from the tissue that had been damaged. The increase in respiration rate was dependent on the extent of wounding, the larger number of cuts and the higher respiration rates (Surjadinata & Cisneros-Zevallos, 2003).
An useful indicator of product physiology is the respiratory quotient (carbon dioxide production/ oxygen consumption) (Kader, 1987) and it was shown that the number of cuts and temperature will increase respiration rates as expected due to enzymatic reactions that may be occurring and promoting oxidation of the tissue. Respiration rates differed according to the type of cut and storage temperature, while ethylene production was not influenced by the type of cut or by storage temperature (Izumi et al., 1996).

3.1.4. Microbial quality

During peeling, cutting and shredding, the surface of the fresh produce is exposed to air and to possible microbial contamination (including bacteria, yeasts and moulds). According to Garg et al. (1990) the

major sources of in-plant contamination are the shredders used to prepare chopped lettuce and cabbage for coleslaw. In particular, in the case of minimally processed vegetables, most of which fall into the low-acid category (pH 5.8-6.0), the high humidity and the large number of cut surfaces can provide ideal conditions for the growth of microorganisms (Willocx et al., 1994).
The entity and dimensions of bacterial populations found on fruit and vegetables vary enormously and depend on a wide variety of environmental and intrinsic variables. Predominant microflora of fresh vegetables/fruits are *Pseudomonas* and *Erwinia* spp., usually with initial counts of around 10^5 CFU/g, although low numbers of moulds and yeasts are also present (Willocx et al., 1994; Varoquaux & Wiley, 1994). During cold storage of minimally processed leafy vegetables, pectinolytic strains of *Pseudomonas* are responsible for bacterial soft rot (Varoquaux & Wiley, 1994). An increase in the storage temperature and the carbon dioxide concentration in the package will shift the composition of the microflora such that lactic acid bacteria tend to predominate (Manzano et al., 1995).
Manzano et al. (1995) concluded that the high level of initial microbial flora of vegetables for soup was probably due to the machinery, the environment, as well as human and natural contamination. Marchetti et a1. (1992) also found high initial counts for psychrotrophic bacteria and total mesophilic bacteria, exceeding even 10^8 CFU/g, in various commercial vegetable salads. Mixed salads and carrots were on average found to be more contaminated than either red or green chicory.
The high initial microorganisms loads in this type of matrixes, makes it hard to establish the cell-number limit beyond which a product will be considered spoiled. A number of works have demonstrated that there is nothing as a simple correlation between chemical or biological single markers (such as pH, lactic acid, acetic acid and carbon dioxide levels and sensory quality, and the total microbial cell load) and spoilage,. Indeed, different spoilage patterns are found for different minimally processed fruit and vegetable products, varying according to the raw materials properties.
During storage, fresh cut packed carrots can be spoiled by growth of lactic acid bacteria that produce lactic and acetic acids (Carling et al., 1990). Non vacuum-packed sliced carrots are susceptible to the growth of *Erwinia* sp. (70% of total flora), *Pseudomonas* sp. (20%) and *Bacillus* sp. (10%). This population evolves after packaging and storage. On the other hand, vacuum-packed carrots exhibit a rather fermentative nature with *Leuconostoc*. as the predominant microorganism genus, which confers a slimy characteristic to the product (Buick & Dmoglou, 1987).
Carlin et al. (1989) showed that cut vegetables were more prompt to spoilage than whole vegetables, as the former release nutrients that support the microbial proliferation.

3.1.5. Cut effects on carrots

Wounding induce a complex series of processes aimed at repairing the damage suffered by the tissue, starting with an immediate response that elicits the biochemical and physiological responses that are needed for the healing routes (Rolle & Chism, 1987; Saltveit, 1997).. The most common responses to wounding include an increased respiration rate, ethylene production, and synthesis or loss of several phytochemicals (Tapadia et al., 1995; Saltveit, 2000). The respiration rate, in fact, is considered an important shelf life indicator of fresh produce. For instance, fresh whole produce with increased respiration rates (Kader, 1986) and wounded tissues with higher respiration rates (Rolle & Chism, 1987) are associated to shorter shelf life. Respiration is thus critical in the design of food packaging, where the targeted atmosphere is often determined using typical respiration rates of non-wounded tissues. This targeted atmosphere might not be preserved in the case of wounded tissues which display increased respiration rates (Saltveit, 1997).
Cutting also affects pH, with usually an increase up to the eight day after the cut process. Even the quality of the blade, i.e., blade sharpness, used for slicing the carrots has been demonstrated to have an effect in the pH of the product during storage. The pH increase coincides with increased microbial population, which in turn may result in larger organic acids utilization (organic acids are consumed, having a consequent increase of pH). King et al. (1991) reported an increase in the pH values over 15

days storage of minimally processed lettuce and correlated it with an increased microbial population.
Wound-induced increased respiration rates have been correlated to greater enzymes synthesis, in particular of enzymes that are involved in the respiration route, e.g., ATP-dependent phosphofructokinase involved in the carbohydrate breakdown that leads to pyruvate and to an enhanced aerobic mitochondrial respiration related to changes in mitochondrial structure and number (Asahi, 1978). Wound-induced respiration can also be partially associated to long-chain fatty acids oxidation from membrane deteriorative processes, wound-induced ethylene, and oxidative reactions leading to browning (Laties, 1978; Rolle & Chism, 1987; Saltveit, 1997). Wounding may also enhance the synthesis of different enzymes in plant tissue (Toivonen & DeEll, 2002) whose induction occurs momentary and could be explained by the presence of an inactivation system used by the tissue to control other enzymes (Creasy et al., 1986). This respiration depends on several factors, namely the temperature, type of tissue, controlled atmospheres, and degree of cutting (Watada et al., 1996; Zhu et al., 2001).
Therefore, considering all these effects, wounding will cause noticeable thermodynamic changes, indeed, by measuring the heat production that follows a cutting operation, it will be possible to assess the wounding response of the tissue. Usually, vegetables under stress can produce up to 1.5 times more heat compared to unprocessed vegetables indicating that the stress condition can boost biochemical reactions due to cell decompartmentation and therefore more enzyme-substrate contact (Wadsö et al., 2004). Other studies dealing with vegetable wounding responses indicate that carrots have certain protective mechanisms to defy stress. This effect was observed when injured carrots cells become stronger by additional lignin secretion as defensive mechanism (Satoh et al,. 1992) or adapted when exposed to low temperatures (Gomez et al., 2004).

3.1.6. Calorimetry and carrots

Carrot and carrot products, either they are fresh or thermally treated, are mainly spoilt by microorganisms. The growth of these microbial populations determines the product shelf-life. One way for obtaining microbiological information more quickly and inexpensively is using accelerated storage, at higher temperatures.
Temperature affects growth conditions and different bacteria can survive according to the storage temperature (Garcia & Zurera, 1997). A drawback of the plate counting approach is cost in terms of material and time and moreover it entails an incubation period of several days. Therefore it is important to consider alternative methods. One of the simplest and most commonly used methods is the pH monitoring, due to the effect that the microbial metabolism exerts on the acid-basic equilibrium of the food matrices. pH monitoring is rapid, though it is not specific method, since other reactions, not related to the action of microorganism, also affect pH.
As illustrated in the precedent sections, all living organisms produce heat because of metabolism. This heat can be evaluated directly in calorimeters and used to assess the level of biological activity. Calorimetric measurements of heat production rate of have been used to provide a direct indication of the metabolic responses of raw materials, such as respiration and reaction to wounding stress (Criddle et al., 1991; Gomez et al., 2004). Additionally, it has been used successfully to evaluate the microbial growth kinetics of in packed, fresh-cut carrots and carrot juice (Riva et al., 2001; Alklint, 2003). A calorimeter is able to measure the overall heat produced which becomes a technique for studying a biological system. Similarly to respirometry, calorimetry does not allow to detail the nature of the changes of the biological system, at least not directly. Nevertheless, an advantage of calorimetry is that it can provide information on metabolic pathways and energy efficiency when metabolic heat, O_2 consumption and CO_2 production are known (Hansen et al., 1997).
Isothermal microcalorimetry represents thus a valid alternative. In this technique, the heat produced in a sample is measured at constant temperature. The thermal power, P, is measured across the time and integrated to find the heat, Q. This technique has showed to be promissory in the study of microbial

activity in complex biological systems (Barja et al., 1997).Calorimetric investigations on microbial systems have been carried out in several applied areas of biology (Wadsö, 1997). The total amount of heat generated is correlated to the processes that take place in the material of interest. The heat produced by microbial-related reactions (aerobic and anaerobic) plus the metabolism of the carrot or the carrot product itself and the associated microbial flora are measured in the calorimeter simultaneously.
During the processing of fresh carrot juice, for instance, carrots are triturated and the the juice separated from the pulp. In pasteurized juice, the liquid is heat-treated at a temperature above the membrane deterioration temperature (Gekas et al., 2000). Therefore only in the particular case of that kind of carrot-derived product, it is unlikely that a significant contribution to the overall heat production will come from the carrot metabolism.
In order to determine the shelf-life, the data obtained from calorimetric measurements can be analyzed different ways. In specific, for isothermal microcalorimetry, the peak heat production time can be determined or the time required for a fixed increase in the thermal power or detection time (Iversen et al., 1989). Otherwise, shelf-life may also be determined considering that the degradation remains of the same order of magnitude as that at the start of storage period. As soon as the microbial growth or the enzymatic degradation reaches the maximum acceleration, this condition does not longer exist.t The maximum/minimum of the second derivative of fitted equations with respect to time can be used for the determination of the shelf-life according to CFU or pH (Riva et al., 2001). This mathematical strategy is valid also for isothermal calorimetry as long as the thermal power is proportional to the total amount of bacteria, N (Barja et al., 1997). In this case, a similar shelf-life is given by the first derivative of the calorimetric trace with respect to time (Riva et al., 2001). The total number of bacteria at a certain time is then proportional to the heat, which can be calculated as the integral of the thermal power until that time.

3.2. AIM OF THE STUDY

The aims of the present study were several: to test calorimetry as a mean for quantifying microbial growth as affected by different cuts of carrots. Calorimetric measurements were carried out on cores taken from carrots cutted at three different ways (cylinder, sticks and Julienne). Since carrots are complex matrices, we choose to investigate the carrot juice and one representative microorganism extracted from carrots, *Pseudomonas fluorescens* sp., as a model.

These objectives were developed through different steps, as follows:.

- Calorimetric evaluation of microbial growth on the three forms (cylinder, sticks and Julienne) with the same weight, and between the same forms with different weights.
- The analysis of the microbial growth, using conventional microbiological techniques, on the three forms (cylinder, sticks and Julienne), with the same weight, and between the same form but with different weights.
- The evaluation of the growth of *Pseudomonas fluorescens* sp. in TSB.
- The evaluation of the growth of *Pseudomonas fluorescens* sp. in carrot juice.

3.3. MATERIALS AND METHODS

3.3.1. Bacterial cultures studied

Pseudomonas fluorescens sp. is a rod-shaped bacterium genus belonging to the family Pseudomonadaceae biotype B. It is ubiquitous in nature, non pathogenic and non-sporulating species; it can grow in temperatures ranging from 4 °C to 40 °C, with emission of fluorescence but not with pigments.
P. fluorescens is aerobic, gram negative, mesophilic but also capable of surviving at low temperature (psychrotrophic), catalase-positive and oxidase-negative. It has pectinolytic, proteolytic and lecithinase activity. It does not have fermentative capability.

3.3.2. Inoculum preparation

The cultivated strain *P. fluorescens* was provided by Pr. Laura Franzetti from Laboratory of Microbiology, University of Milan. Frozen storage cultures of *P. fluorescens* were then pregrown on Petri dishes with TSA for 24 h at 31°C. One colony from a pregrown Petri dish was used as an inoculum for a 10 mL culture in liquid TSB at 31°C. 1% of bacterial suspension grown overnight (exactly 24 hours) was used as inoculum for the next liquid 10 mL TSB culture and grown 24 h which allowed the *P. fluorescens* to reach the middle of the exponential growth phase. The number of bacteria was determined by plating on TSA and incubating for 2 days at 31°C, and average number of bacteria in the mid-exponential culture was determined $(4.15 \pm 0.15)*10^8$ CFU/mL.

3.3.3. Growth experiment

Medium composition for *P. fluorescens* was TSB (tryptic soy broth), see Table 3.3. Solutions of TSB were prepared mixing 30 grams in 1 L of deionized water and the pH was adjusted to 7 and then sterilized by autoclaving at 121 °C for 15 min.
The growth of *P. fluorescens* in carrot juice was made in the same conditions as in TSB, adjusting the pH of the juice carrot to pH 7 with lemon juice; the initial biomass concentration was in both cases of experiments in a range of 500 to 600 CFU/mL. Experiments were carried out in 15 flasks containing 5 mL medium volumes. The culture temperature was 30 °C (the same as the temperature of calorimetric runs).
Biomass concentration was measured by counting cell colonies (CFU/mL) on TSA plates. The plates were incubated for 48 h at 30°C.

Table 3.3. The composition of the culture media TSB

Component	Amount
Enzymatic digest of casein	17.0 g
Enzymatic digest of soybean meal	3.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Dextrose	2.5 g

3.3.4. Instrumental: Isothermal Calorimetry (IC)

The growth of microorganisms in cutted fresh carrots and the growth of *P. fluorescens* in TSB and in carrot juice was performed with a calorimeter E. Calvet Micro-calormétrie, DAM (Setaram, Lyon, France) at 30 °C, equipped with two suitable cells of 13 mL. A first calorimetric cell was used as

reference and filled with inert material, while the second one, previously sterilized, was filled up with 5.0 and 6.6 grams of carrot cuts prepared previously immediately before every run. Carrots were first washed to make sure the carrot tissues were free of mud particles and other extraneous material. The carrots were then peeled using a sterile manual peeler.
In the case of the study of the microbial growth of *P. fluorescens* in TSB and in juice carrot, the calorimetric cell was previously sterilized and filled up with solution containing 5 mL medium volumes (carrot or TSB). Isothermal calorimeter traces were obtained at 30°C. Calibrations were performed at the end of every experiment (0.1 mW*1200 s) using the Joule effect calibrator EJ2 (Setaram, Lyon, France).
The heat flux-versus-time raw signal was numerically integrated to obtain the overall specific enthalpy ΔH/J g and/or ΔH/J mL. Three replicates were performed for each experiment. The ΔH errors were minor to 10%.

3.3.5. Preparation of samples

3.3.5.1. Plant material

The whole carrots used in the experiment were obtained from a local producer from Northern Italy. They were then sorted manually based on their size, washed with hydrogen peroxide (1 %), then hydro-cooled to about 4 °C and stored in one large polyethylene plastic bag at 0°C and approx. 100 % relative humidity, as routine practiced by the producer. Carrots were first washed to make sure that carrots were free of mud particles and other extraneous material. Medium-sized carrots (19.5 +/- 1.0 cm in length, 3 +/- cm diameters) free from defects, were selected at the farm storage facility and transported to our laboratory in the plastic bag kept in ice in insulated boxes. The carrots were then taken out of the bags and placed in a closed, refrigerated chamber at 0.2 °C and 100 % relative humidity until used for experiments.

3.3.5.2. Cut processing

The carrots were then peeled using a sterile manual peeler, the top and tip removed and the remainder cut into cylinders (13 mm of diameter and 6 cm in length), "Julienne" slices (2 mm of thickness and 6 cm in length) and sticks (2 mm of thickness, 12 mm of width and 6 cm in length) (Figure 3.1).
The samples have around 6.6 +/- 0.1 g and the cut operations were done in sterility, the calorimetric cell was sterilized before every experiment.
The juice was made from hand-peeled carrots using a household juice machine. The pH of all samples was 7 +/- 0.1, and with the addition of lemon juice it was taken down to 4.5 +/- 0.1.
For the calorimetric experiment, 5 mL of carrot juice were introduced into 12 mL stainless steel cell. All samples were prepared immediately before the analysis in the calorimeter.

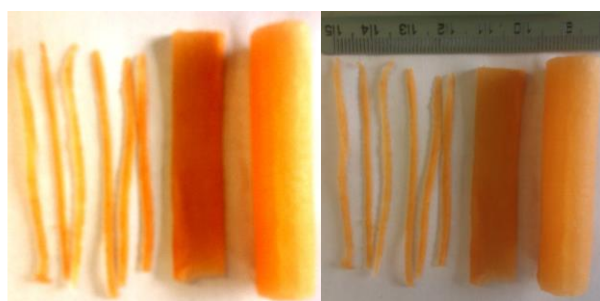

Figure 3.1. Samples of cut carrot (sticks, cylinder and Julienne).

3.4. RESULTS AND DISCUSSIONS

3.4.1. *P. fluorescens* growth

The main aim of this investigation was the search of a suitable protocol to check the microbial growth in fresh carrots that underwent different kinds of cutting and no other treatment.
Usually such systems host a random starting microbial contamination, which is the result of the endogenous microbial flora joined by other microbes from the environment and/or any experience treatment of the raw product. Because of this, any direct match between samples obtained from different lots is largely unreliable.
On the other hand, the usual microbial flora of carrots is well known and investigated (see above): one typical microorganism is *P. fluorescens*. This was used as a reference for the present investigation.
Cultures of this microbial species were realized in either TSB medium or in carrot juice. Figure 3.2 reports the calorimetric traces obtained from two samples of cultures in TSB medium.
A main large exothermic signal reflects the growth of the microorganism, which, in the conditions considered, namely, 600 CFU/mL starting population, shows the onset at about 10 hours. Comparison of these data with the plate count results (Figure 3.3) allows one to recognize that the onset of the calorimetric signal corresponds to the attainment of a $10^{5.5}$ CFU/mL. Such detection threshold was taken into account in the investigations related to the microbial growth in carrots.
To garner a further reference, the same microorganism was allowed to grow in carrot juice at pH 7, i.e., close to the real condition of carrots.

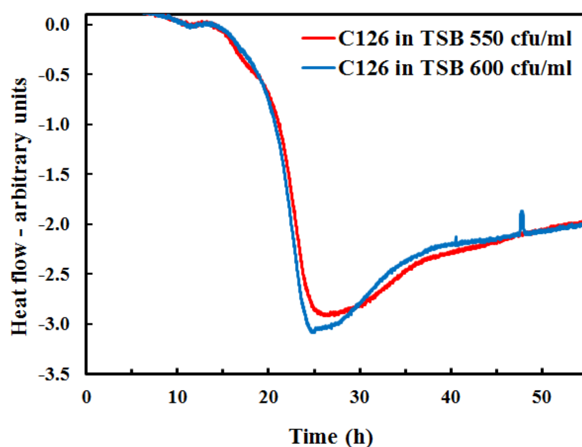

Figure 3.2. Power–time curves of the metabolic activity of *Pseudomonas fluorescens* with different inoculation rates in TSB.

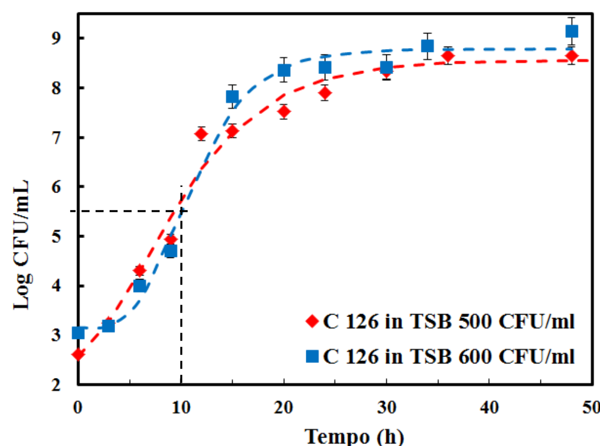

Figure 3.3. Microbial growth of *Pseudomonas fluorescens* with different inoculation rates in TSB (plate count).

These calorimetric results were compared with those collected from a culture in non-modified carrot juice. Figure 3.4 shows the match between the calorimetric traces.

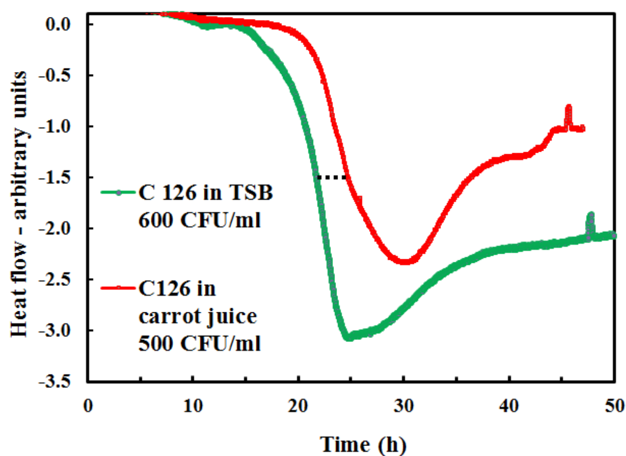

Figure 3.4. Power–time curves of the metabolic activity of *Pseudomonas fluorescens* in TSB and in juice carrot.

The signal observed is rather similar in shape, although of smaller intensity with a slightly delayed onset. The match with the corresponding plate count data (Figure 3.5) confirms the shift of the onset and allows one to verify that the detection threshold as for the microbial population is the same.
This conclusion allowed us to use the onset of the calorimetric traces obtained from real systems (carrot undergone different cutting treatment) to normalize the relevant microbial contamination with respect to a common starting level, namely, the detection threshold so far determined. The onset point was therefore selected as the starting point and set at zero time.

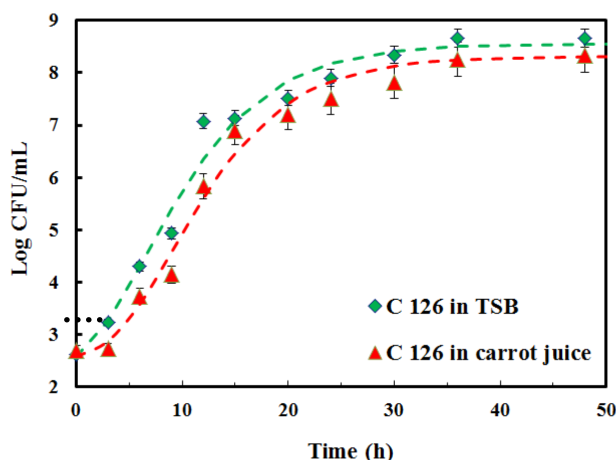

Figure 3.5. Microbial growth of *Pseudomonas fluorescens* in TSB and in juice carrot (plate count).

3.4.2. Cut effect on carrot

Figure 3.6 shows the comparison of the calorimetric traces obtained from fresh carrots that had undergone a different cutting treatment, i.e., cylinder, sticks, Julienne. All these signals reflect both the growth and the metabolism of the overall microbial population. In spite of the expected differences between the microbial populations present in the three kinds of carrots, the signals observed are rather similar to one another and to the signal of the reference system. This evidence seems a support for the adopted normalization criteria.

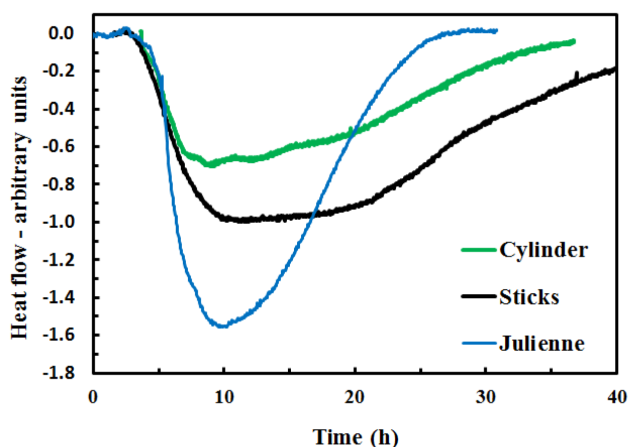

Figure 3.6. Power–time curves of the metabolic activity of different kind of cut fresh carrot.

The rate of microbial growth and metabolism is larger for the carrots cut *à la Julienne* than for carrot sticks and carrot cylinders, although the starting growth rate is similar for the three kinds of cutting. After the early phase, the lower the exposed surface (that depends on the cutting kind) the smaller the microbial growth rate observed.

The amplitude of the peak signal is different for the three samples and this due to the different metabolic activity and the growth rate of the microbial population.

The overall enthalpy drop changed for all three kind of cut and increase with the increase of the area

exposed to the cutting process (see the values reported in Table 3.4).

Table 3.4. Carrots and carrot juice enthalpy (ΔH) from power–time curves.

Sample	ΔH / mJ.g^{-1}
Julienne	5300
Stickes	5000
Cylinder	2700
Carrot juice	5100

Figure 3.7 shows the metabolism of *Pseudomonas fluorescens* (5 10^4 UFC/mL) growth in TSB medium studied by both the IC and plate count. As illustrated form the two relevant curves, the log phase of the IC curve of *P. fluorescnes* is in a satisfactory agreement with that of the plate count. As mentioned above, the IC data provide more details about the growth process.

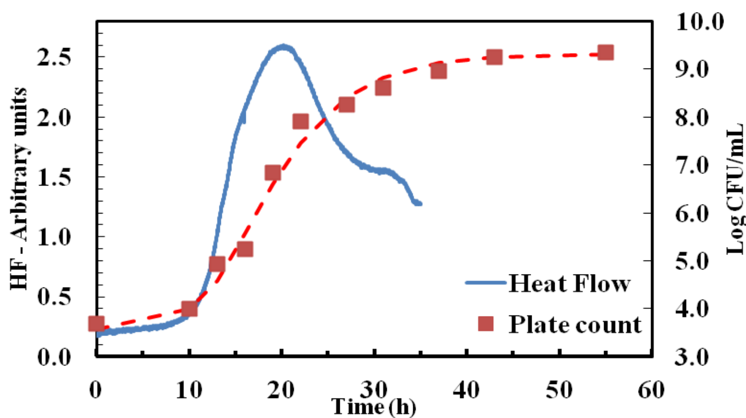

Figure 3.7. Heat flow trace (left vertical axis) and plate count (right vertical axis) of *Pseudomonas fluorescens* in TSB.

3.5. CONCLUSIONS

The approach allowed the assessment of a protocol to overcome the problem of the unknown starting level and the heterogeneity of the microbial population hosted in the carrot samples and the evaluation of the growth rate with simple isothermal calorimetry investigations. Details about the specific mechanisms underlying growth and metabolism are out of the scope of the present research, but it seems that calorimetry may be of help to characterize the microbial contamination in products of this kind.

Details of the present investigations will be reported in a ready to submit paper.

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4. ISOTHERMAL CALORIMETRY APPROACH TO EVALUATE SHELF LIFE OF READY TO EAT SALAD

4.1. STATE OF THE ART

4.1.1. Corn salad

Corn salad (*Valerianella locusta* Laterr.) is a small dicot annual plat of the family Valerianaceae. It is an edible green salad with a characteristic nutty flavor, dark green color, and soft texture. Common names include Lewiston cornsalad, lamb's lettuce, mâche, fetticus, feldsalat, nut lettuce, field salad, Nüssli Salat, and rapunzel.
It is largely consumed in salads for its good taste and dietary characteristics, grows wild in parts of Europe, northern Africa and western Asia. In Europe and Asia it is a common weed in cultivated land and waste spaces. In North America it has escaped cultivation and become naturalized on both the eastern and western seaboards.
The narrow, dark green leaves are tender and have a tangy, they can 15.2 cm long. Usually are consumed as fresh products and for this are subjected to minimal processing to preserve their freshness. They belong to the convenience foods category "ready-to-use vegetables", as they offer many added features like freshness, commodity of use, good retention of nutritional qualities (loaded with beta-carotene, a pigment the human body can convert to vitamin A), like other formerly foraged greens, corn salad has many nutrients, including three times as much Vitamin C as lettuce, beta-carotene, B6, B9, Vitamin E, and omega-3 fatty acids (Giovenzana et al,. 2014; Kolton & Baran, 2008; Ferrante et al., 2009)
These products are low-processed; they are picked, washed, dried (centrifuged) and packed then commercialized ready to eat without further handling (Baur et al., 2005; Della Rosa & Ruccoli, 2007). The above operations can increase the respiratory activity of the leaves, causing negative effects on their texture and color, and rendering them more perishable than corresponding fresh vegetables. The stabilities of these products depend on the qualities of raw materials, the handling procedures, the packaging modes, and the storage conditions (Morgante et al., 2008; Legnani & Leoni, 2004).

4.1.2. Minimally processed corn salad

The relevant operations (processes) for an accurate final quality of the product are:

Washing: The objective of washing is to remove exudates from the leaves as well as soil and other possible contaminate, and to reduce the temperature of the produce (Kader & Saltveit , 2003). It is interesting that sanitizing agents such as chlorine are allowed and used in the wash water, mainly to reduce the microbial load in fresh-cut vegetables (Soliva-Fortuny & Martin- Belloso, 2003).
However, some manufacturers are at times directed to use chlorine and warm water in the washing step for the reduction of browning (Baur et al., 2004).

Drying: the role of drying is to remove the excess of water retained by the product during washing (Moretti et al., 2007). Drying by centrifugation is widely used in fresh-vegetables industry, but also other methods can be used for water removal such as vibration screen and forced air tunnel (Moretti et al., 2007). For ready-to-use products, it has been reported that slight desiccation improved the shelf-life (Bolin & Huxsoll, 1991). However, an over-centrifuged product can increase the mechanical damage in the tissue i.e. cracks and crush, speeding up the loss of quality (Moretti et al., 2007).

Packaging:
Modified atmosphere packaging (MAP) has been successfully used in ready-to-eat products to reduce browning by creating an atmosphere with low quantity of O_2 and high quantity of CO_2 (relative to air) and by storing at specific cold temperature (Martin-Belloso, 2003) and to reduce the salad respiration rate, with the benefit of delaying senescence thus extending the storage life of the fresh produce and

reducing salad metabolism and maturation (Kader et al., 1989).
The potential of MAP to extend shelf-life for many foods has been well documented (Brecht et al., 2003; Jacxsens et al., 2003).

4.1.3. Effect of minimal processes on fresh cut vegetables

Quality of fresh vegetable products determines the value to the consumer and is a combination of parameters including appearance, texture, flavor, and nutritional value (Kader, 2002).
Shelf-life is the time before the product attributes drops below the acceptance limit under standardized storage conditions (Tijskens, 2000).
All the operations that the product is undergone from the cultivation to the use can affect its quality and its safety specially:

- washing or sanitizing with the aim to remove the dirt and to reduce microorganisms responsible for quality loss and decay (Sapers, 2003), usually using liquid chlorine or hypochlorite in concentration levels of 50 e 200 ppm free chlorine and with typical contact times of less than 5 min (Francis & O'Beirne, 2002; Watada & Qi, 1999).
- or wounding that can cause physiological effects, includine ethylene production, increase in respiration, membrane deterioration, water loss, susceptibility to microbiological spoilage, loss of chlorophyll, loss of acidity, increase in sweetness, formation of flavor volatiles, tissue softening, enzymatic browning, lipolysis and lipid oxidation (Toivonen & De-Ell, 2002).

4.1.3.1. Colour evaluation of minimally processed vegetables

Color is one of the main attributes, along with texture, that characterizes the freshness of most vegetables. This characteristic can change under minimal processing on corn salad due to different biochemical processes, mainly chlorophyll degradation and browning appearance, Browning of fresh vegetables reduces quality (Shewfelt, 1994) and is often the factor limiting shelf-life and marketability of fresh-cut lettuce (Lopez-Galvez & Cantwell, 1996). This can be explained due to the de novo biosynthesis of polyphenols (Minoura & Homma, 2004).
Chlorophyll is also tightly correlated with carotenoids because these later have to protect the chlorophyll from photo-oxidation during growth (Biswall, 1995).
During postharvest life the leaf pigments undergo degradation that leads to leaf discoloration. Total chlorophyll and carotenoid contents start to decline a few days after harvest. This phenomenon has been observed in many leafy vegetables. Among carotenoids, β-carotene, which is the most important antioxidant in vegetables, is the most sensitive component to degradation (Negi & Roi, 2004).
During the storage, the chlorophyll amount decreased significantly after 5 - 8 days, and this is longer than the normal selling period of 5 - 6 days in most cases. Total phenols variability suggests that inter-conversions among the many groups of polyphenols may occur (Ferrante & Maggiore, 2007).

4.1.3.2. pH variation

Corn salads belong to the low-acid foods (pH 5.8 - 6.0) (Willkox et al., 1993), however this parameter can change after minimally processed vegetables processing or by the storage conditions at different temperatures due to the microbial metabolic activity (i.e. organic acid or CO_2 production). The pH of the vegetables studied is considered to be adequate in a range of 5 - 6.5 for quality retention. The storage temperatures affect not only the gas concentration inside the salad bag or the number of microbial population but also the pH. The CO_2 concentration at the temperature of storage 15°C (52.67%) seem to be the cause of the decreasing of the pH from the starting value (near to 7) to the finale value (near to 4) (Beuchat, 1992).

4.1.3.3. Microbiology of minimally processed vegetables

The natural microflora of fresh-cut vegetables includes bacteria, yeast and moulds. The load of the natural microbial depend directly to the type of salad composition, environment considerations, seasonality, initial contents of raw material, and the conditions under which a particular vegetable is grown (Francis et al., 1999). The minimally processed vegetables bear a great influence on the microbial concentration and can be cause of microbial spoilage or contamination by increasing the moisture content, or increasing the active metabolism of plant tissue. The impact of microorganisms on the quality of the final product by the decreasing of the shelf life through spoilage (Nguyen-the & Carlin, 1994).
The species most frequently present in the corn salad were *Pseudomonas fluorescens* (19%) especially at the beginning of the shelf life, followed by *Pseudomonus putida* (8%). This predominance of the genus *Pseudomonus* among the psychrotrophic bacteria has been described elsewhere (Marchetti et al., 1992; Willocx et al., 1993). Moulds can be also detected in the salads but not always, possibly due to the modified atmosphere in which the CO2. The effect of temperature of storage on the growth of micro-organisms was evident.
At the end of the shelf-life it was noted the presence of Enterobacteriaceae, less psychrotrophic, increases especially in the product stored at 10°C encouraged by the packaging under Modified Atmosphere. Their number increases during the storage and at the end of shelf-life, they become the prevalent microbial population (Caldera, 2013).

4.1.3.4. Nutritional content of minimally processed vegetables

The minimally processed salad during harvesting, processing and handling can affect the nutritional value of the product specially antioxidant status (Lindley, 1998) and consequently its sensorial characteristics. I many studies it was found that the processing can decrease the antioxidant activity in the green fresh products as in fresh cut spinach (Gil et al., 1999), or in Iceberg and Romaine lettuce (Kang & Saltveit , 2002).
The decrease of the antioxidant activity can be quantified by the measure of the amount of ascorbic acid, carotenoids or polyphenols (Lana & Tijskens, 2006), this decrease is usually preceded in the first time immediately after the processing by one increase of the antioxidant capacity content (Zhan, 2009)
Total phenols had the same trend with oscillations during storage. Lower values were found in leaves stored at 4°C at the end of the storage period. (Ferrante & Spinardi, 2012)
The total phenolics content was significantly influenced by the interaction between the harvest handling and storage condition over time and increase during the first phase of storage reaching its maximum value followed by a gradually decrease in the second phase of storage (Zhan, 2009).

4.1.4. Isothermal calorimetry and corn salad

IC is potentially a useful tool for shelf-life predictions. Several attempts have been made in this direction. For example this technique was used to predict the shelf-life of salads (Riva et al., 2001).
Alklint et al., 2005 used this technique in parallel with plate counts to quantify microbial spoilage of carrot juice depending on the temperature of storage, relying on the fact that plate counts is the standard method able to give all the microbial influence on the product, they considered that calorimetry could possibly be used as a complement to plate counts since the microbial growth produces heat. This technique was also successfully used in the case of fresh cut carrots to monitor the growth kinetic of endogenous microbial species (Riva et al., 2001).

4. Isothermal calorimetry approach to evaluate shelf life of ready to eat salad

Corn salad as all living systems produce heat as a result of metabolism, this heat can be detected directly with isothermal calorimeter and used to assess the level of biological activity since all chemical reactions and biological processes are accompanied by the generation or absorption of heat. The calorimetric measurement of the rate heat production can be used to provide a direct indication of the metabolic activity of raw material, such as respiration and reaction to wounding stress (Gomez et al., 2004).

4.2. AIM OF THE STUDY

The aims of the present study were several:

- To test calorimetry as a technique to monitor the shelf life of corn salad at different temperature of storage. Therefore, calorimetric measurements were carried out on samples taken from corn salad stored at three different temperatures (5°C, 10°C and 20°C); the choice of these three values of temperature was made considering that 5°C is the ideal temperature of storage, 20° is the extreme condition that can accelerate all the enzymatic reactions and 10°C can be the real temperature of storage (Jacxsens et al., 2001).
- To evaluate the impact of the temperature of storage on the product by the use of different parameters such:

- Microbial growth by plate count
- pH variation during storage
- samples moistures trend during storage
- color changes of samples during storage
- the antioxidant activity by the change of polyphenolic quantity
- Two different kind of isothermal calorimeters were used, the first one to monitor the metabolic activity during the storage at three different temperatures and the second one to assess the impact of the temperature of storage on the metabolic activity by the analysis of the metabolic activity at 30°C.

4.3. MATERIALS AND METHODS

4.3.1. Preparation of samples

Pouches of 100 g of ready-to-use corn salad (*Valerianella locusta* Laterr.) were obtained from a local farm near Milan (Italy), packaged in modified PVC. The packages were not airtight and the wrapping material (15 µm thickness) was highly permeable (typical permeability at 06 °C, data from the film producer - was 80000 cm^3/m^2 24 h bar for CO_2 and 15000 cm^3/m^2 24 h bar for O_2). Packaged salad leaves came from the same producer (see figure 4.1), were transported, immediately after packaging, under refrigerated conditions at 4 °C to our laboratory: the arrival time was considered the zero time (t_0) for all the kinetic evaluations, and subjected to microbiological and chemico-physical analysis upon arrival. Shelf-life tests were performed by storing the products in controlled conditions (5, 10, and 20°C), the relative humidity was 82%. The measurements of shelf-life indices were performed every day at 5°C and at 10°C, and every day for seven days for 20 °C (± 0.5 °C).
The analyses of corn salad were made in the following days after the production (Table 4.1).

Table 4.1. Sampling points during shelf-life monitoring for 5°C, 10 °C and 20 °C.

Sample code	Storage time (days)	Storage temperature (°C)
T0	0	5-10-20
T2	2	5-10-20
T3	3	5-10-20
T4	4	5-10-20
T5	5	5-10-20
T6	6	5-10-20
T7	7	5-10-20
T9	9	5-10
T10	10	5-10
T11	11	5-10
T12	12	5-10
T13	13	5-10
T14	14	5-10

Figure 4.1. Corn salad leaves and packed corn salad.

4.3.2. Microbiological analysis

Tens grams of samples from each package were drawn and homogenized with 90 mL of sterile trypton salt solution at 0.85%, into a sterile Stomacher bag, by the use of a Colworth 400 Stomacher for 2 min. Decimal progressive dilutions were prepared and the following bacteriological determination was carried out, mesophilic aerobic bacterial count or Total Bacterial Count (TBC) (Iso 4833, 2003) on Tryptic Soy Agar (TSA, Merck, Germany) (APHA, 1992) spread plates, incubation at 30 °C for 48 hours.
All microbiological analyses were carried out in triplicate, and the results were expressed as the mean CFU per gram.

4.3.3. Color determination

Chlorophyll a fluorescence (expressed in relative units) was determined on dark adapted leaves randomly taken from the stored boxes and incubated for 40 min at room temperature, using a portable Handy PEA (Hansatech, Norfolk, UK). The measurements were taken on the leaf surface (4 mm diameter) exposed to an excitation light intensity emitted by three diodes. Leaf fluorescence detection was measured by fast response PIN photodiode with RG9 long pass filter (Technical manual, Hansatech). The parameters measured were Fo, Fm, Fv⁄Fm. The Fo represents the fluorescence level when the plastoquinone electron acceptor pool (Qa) is fully oxidized. Fm represents the fluorescence level when Qa is transiently fully reduced.
The Fv⁄Fm ratio represents the maximum quantum efficiency of photo-system II, the Fv is the variable fluorescence (Fm–Fo).

4.3.4. Total phenols extraction and determination

Phenolic compounds were extracted from 10 g of leaves using 60 mL of methanol (100%). The samples were homogenized by Ultraturrax (IKA T25 digital) and covered with aluminum paper in agitation for 30 min, were centrifuged controlling the temperature for 10 min at 6000 rpm for removing solids. (Temperature was 20°C ±1) the last operation was made two times adding 60 ml of methanol (100%) (Vina & Chaves, 2006).
Phenols in the extracts were determined using the Folin–Ciocalteu reagent (Sigma, Milan, Italy) (Singleton et al., 1999). Readings of reactions containing 200 mL extract and 0.5 mL Folin–Ciocalteu reagent plus water up to 10 mL were performed at 760 nm using an UV–vis spectrophotometer (Evolution 300, Thermo Scientific, Rozzano Italy) after 2 h incubation at room temperature.
The result was expressed as milligram gallic acid per gram of fresh weight (mg gallic acid g^{-1} FW) (Ke & Saltveit, 1989)

4.3.5. Moisture and pH determination

Samples of 20 g from each package were shopped and homogenized OMNI MIXER 17106 (Sorval, USA) with 40 mL of distilled water for 5 min and successively filtrated with filter paper. The pH was performed on the homogenate by a digital pH meter Radiometer Analytical Ion Analyzer mod. 450.
The analyses were carried out in triplicate, and the results were expressed as the mean of the pH values.
Five grams of corn salad were weighed by Sartorius MA150 Moisture Analyzer, dried at the temperature of 105 °C until constant weight, the analysis were carried in triplicate and the results were expressed as the mean of % values.

4.3.6. Calorimetric measurement

IC was performed firstly with “Calorimètre E. Calvet pour Micro-calormétrie, DAM” (Setaram, Lyon, France) at 30 °C, equipped with two suitable cells of 13 mL volume. A first calorimetric cell was used as reference and filled with inert material, while the second one previously sterilized was filled up with 2 grams of product at every run. The isothermal calorimeter traces for the different temperatures were obtained at the running temperature 30°C.
The second calorimetric measurements were performed by Thermal Activity Monitor 2277 (TAM) from Thermometric (Stockholm, Sweden) (Fig. 4.2), equipped with 20 ml ampoules.
Two grams of salad leaves were placed in the sample ampoule previously sterilized whereas the reference ampoule was empty. The instrument was pre-equilibrated at 10 or 20 °C depending on the experiments. Each experiment was performed thrice to guarantee the reproducibility of the results.

4.3.6.1 The TAM calorimeter

TAM (Figure 4.2) is a multichannel microcalorimeter designed to monitor a wide range of processes over the temperature range 5-80°C (±0.0002 °C). Continuous heat flow measurements are conducted in an isothermal system where thermal events producing fractions of 1 μW can be detected. This level of detectability is achieved by high precision thermostating and sensitive detectors.
The thermal stability is achieved by utilizing a 25 liter water thermostat which surrounds the reaction measuring vessels and act as an infinite sink. Several interactive controlling system work together to maintain the water at a constant temperature. Up to four individual measuring vessels can be housed in the water thermostat and the heat produced or consumed by a sample in the reaction vessel is channeled through highly sensitive thermopile blankets (Peltier elements). These detectors convert the heat energy into a voltage signal proportional to the heat flow.

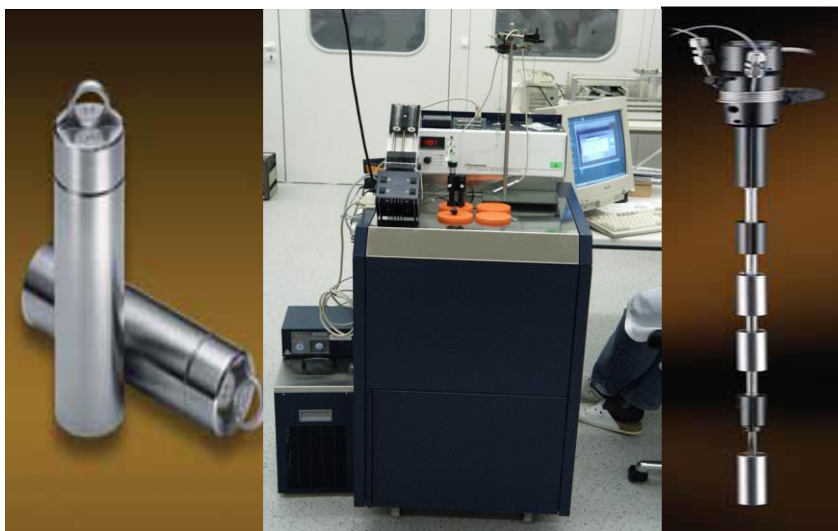

Figure 4.2. Isothermal calorimeter TAM (center), calorimetric cells (left) and Injection system (right)

4.4. RESULTS AND DISCUSSIONS

4.4.1. Calorimetric measurement

Figure 4.3 shows the calorimetric traces obtained at 10 and 20°C from fresh salad samples hosted in the TAM calorimeter (the signals are reverse with respect to previous pictures).
The signal recorded at 10 °C reflects, as expected, a slowed process, although the growth extent can attain a final end plateau, as in the case of the culture monitored at 20 °C. The end tail of the signal reflects the microbial metabolism not coupled with cell duplication.

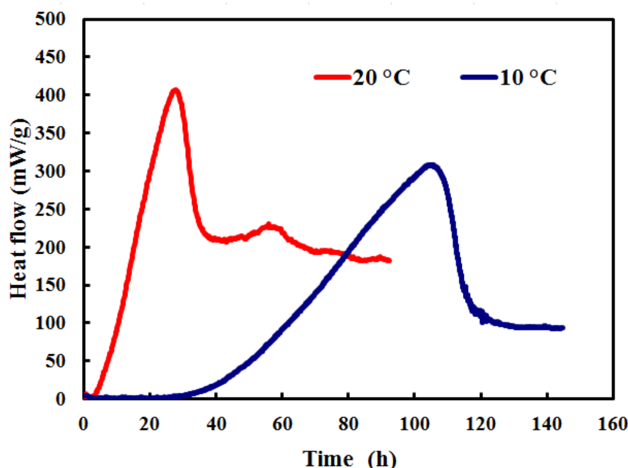

Figure 4.3. Calorimetric power-time curves of corn salad at 10 and 20 °C recorded with TAM.

Plate count data (see Figure 4.4) indeed show that the microbial growth attains a plateau rather quickly (about 4 days) and cell population remains unaltered after on (14 days).
However the fresh salad undergoes some deterioration (chemical, textural, etc.) during such a long storage. In the present investigation the steady level attained by the microbial population after a 4 day storage was used as a biosensor to check the modifications occurred in the salad leaves.
To this aim, salad samples that had been stored for different time lapses at 10 and/or 20 °C, were calorimetrically investigated in isothermal conditions at 30 °C. This temperature rise was intended to stimulate the microbial metabolism, which would however be activated in different matrices according to the extent of deterioration experienced during the previous storage.
Figure 4.5 shows the calorimetric traces relevant to salad samples that had been stored at 10 °C for 4, 9 and 11 days.
The calorimetric traces relevant to more aged samples show signals with larger intensity. This means that the damage experienced by the salad leave facilitates the microbial growth. A more accurate inspection of the data allows one to recognize that such enhancement of the microbial activity is actually the result of a combined effect of temperature and time of conservation.

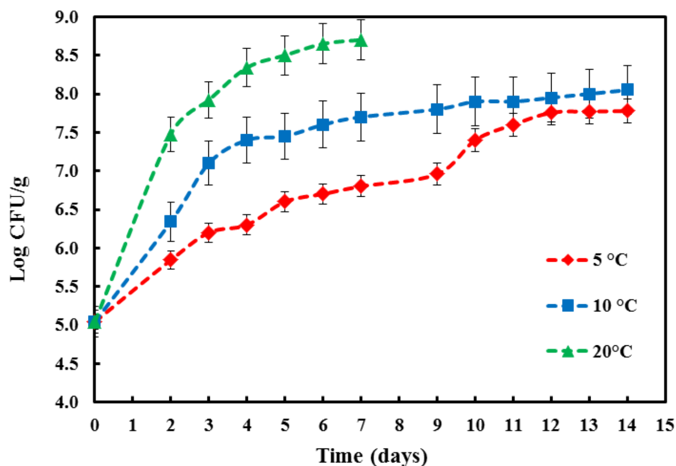

Figure 4.4. TBC trend in corn salad stored at 5, 10 and 20 °C.

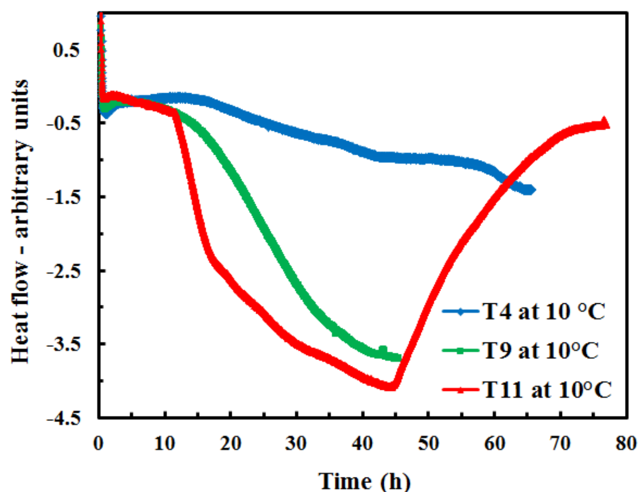

Figure 4.5. Calorimetric power-time curves of corn salad stored at 10 °C after 4/9/11 days of storage, run temperature 30 °C.

Figure 4.6 shows that salad stored at 5°C for 14 days has a behavior similar to that stored at 10°C for 11 days.

It must be once more stressed that the onset of the calorimetric signal occurs at about the same time for all these samples. Which means that the starting microbial population is practically the same in all cases.

The calorimetric approach is therefore adequate to characterize the ageing of the product in the framework of a TTT (Time, Temperature, Transformation) diagram.

In order to single out the factors that make the salad leave a more medium for the microbial biosensor, the evolution of some other parameters was monitored during the storage life at various temperatures.

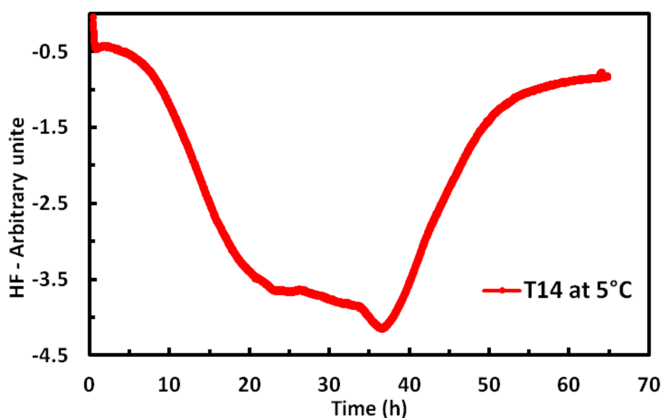

Figure 4.6. Calorimetric power-time curves of corn salad stored at 5 °C after 14 days of storage, run temperature 30°C.

It must be once more stressed that the onset of the calorimetric signal occurs at about the same time for all these samples. Which means that the starting microbial population is practically the same in all cases.
The calorimetric approach is therefore adequate to characterize the ageing of the product in the framework of a TTT (Time, Temperature, and Transformation) diagram.
In order to single out the factors that make the salad leave a more medium for the microbial biosensor, the evolution of some other parameters was monitored during the storage life at various temperatures.

4.4.2. Chlorophyll a fluorescens

Figure 4.7 shows the chlorophyll *a* index, which is used to monitor the shelf-life of the salad. This index is calculated as the ration between the maximum value of fluorescence; F_m, and variable fluorescence, F_v, drawn from the fluorometric analysis (Ferrante & Maggiore, 2007).
The (F_m / F_v) ratio is related to the quality of the salad leave (Schofield et al., 2005) and shows a decreasing trend during the storage period.

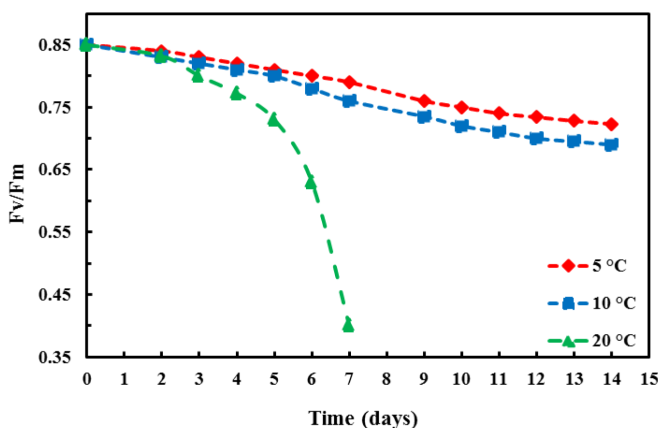

Figure 4.7. Evolution of chlorophyll *a* index trend in corn salad stored at different temperatures.

Figure 4.7 data concern the (F_m/ F_v) trends detected at various temperatures. As expected, a steeper trend of the chlorophyll *a* index was observed for salad stored at 20°C. The trends relevant to the other two storage temperatures (5 and 10°C) become different from each other (the steeper is that for the salad stored at 10°C) only for a storage time longer than 4 days, which is the suggest shelf life for this product. For a shorter storage time, even the product kept at 20°C cannot be reliably distinguished from those kept at 5 or 10°C.

4.4.3. pH evolution

Ageing of corn salad can followed through the trend of its pH, which is an easy to detect and reliable index of either the textural degradation or the microbial spoilage.
The microbial metabolism implies the consumption of sugars, organic acids with the production of catabolites which contribute to raise the pH value (Soliva-Fortuny et al., 2004). The microbial strains responsible for such change are GRAM negatives microorganisms and mostly belong to the species of *Pseudomonas*.
Figure 4.8 reports the pH trend observed for salad leaves stored at the three temperatures considered in this investigation.

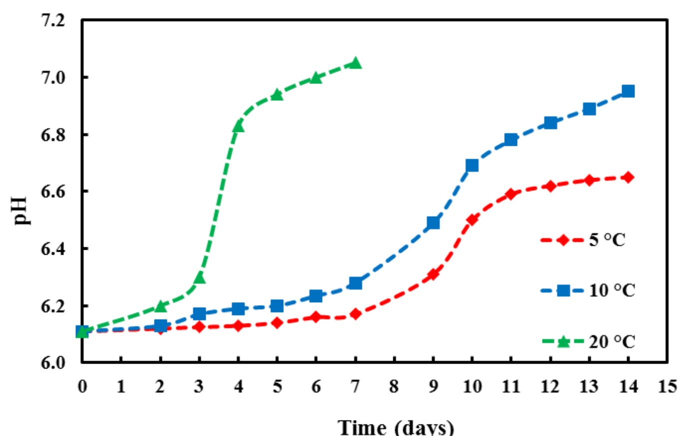

Figure 4.8. pH trend in corn salad stored at different temperatures.

All samples show an pH increasing trend which is steepest for the samples stored at 20°C.
At this temperature, the corn salad attains a pH value of 6.9 after 4 day storage, while salad stored at 10°C attains such pH level only after 12 days, namely, much beyond the recommended shelf life.
These data were treated as directly proportional to the microbial growth and used for a best fit through the Gompertz equation:

$$N= N_0 \exp\{a \exp [- \exp (b - c t)]\}$$

which is currently used to fit the sigmoid trend of the growth of microbial cultures (Zwietering et al., 1990), so as to evaluate the lad phase, λ= (b-1/c), the maximum growth rate, μ = a c/*e* (*e* being the basis of natural logarithms), the maximum microbial population, N_{max}= N_0 e^a (N_0 being the starting microbial population).
In the present case, the main focus was addressed to the following parameters the values of which are reported in Table 4.2. The fitting approach was performed with Table Curve (version 4, Jandel Sci., Erkart, Germany) software.

- log N_∞/N_0 (N_∞ being the final microbial population)
- λ (lag phase)
- μ (maximum growth rate)

These data show that λ, namely, the lag phase period during which the pH remains unchanged, is rather similar for all the three temperatures considered. As expected the maximum growth rate strongly depends on the temperature: At 20°C the maximum growth rate (6,747 d^{-1}) is about six time larger than at 5 and 10°C (1,014 and 0,64 d^{-1}, respectively: these two values must be considered not significantly different from each other).
Since the starting microbial population (10^5 CFU/g) and its maximum allowed value in the fresh product are known , one can determine the limit shelf life of the product at the three temperatures considered, namely, one day at 20°C and 6 days at 5 and 10°C.

Table 4.2. Gompertz coefficients for corn salad stored at 5, 10 and 20°C.

T(°C)	Log N∞/N0	λ	μ max	r^2
5	6,119	9,354	1,014	0,999
10	6,120	5,410	0,64	0,997
20	6,145	2,929	6,747	0,967

4.4.4. Evolution of polyphenols

The content of polyphenols (expressed as mg of gallic acid per 100 g of sample) changes during the storage showing a bell shaped trend. Figure 4.9 shows the trends observed at the three temperatures considered in this investigation.
At 20°C (371,722 mg/100 g) is attained after 4 days. The following decreasing trend is due to the oxidation process.
At 5 and 10°C the maximum concentration is attained after 7 and 9 days, respectively.

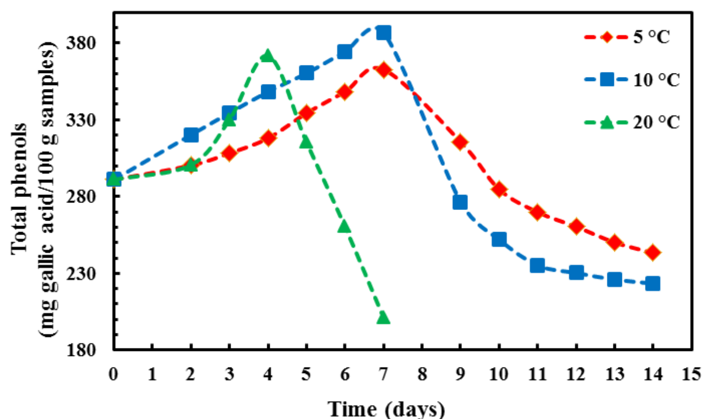

Figure 4.9. Total phenols trend in corn salad stored at different temperatures.

4.4.5. Moisture evolution

Figure 4.10 shows the loss of the moisture content during the shelf life of the salad stored at the three temperatures considered.

Dehydration never exceed 1% of the starting weight no matter the storage temperature considered, although the largest process rate is observed at 20°C. A straight line fit of the data allows evaluation of the corresponding dehydration rate, namely, 0.0013, 0.0006, 0.0005 d^{-1}, at 20, 10 and 5°C, respectively.

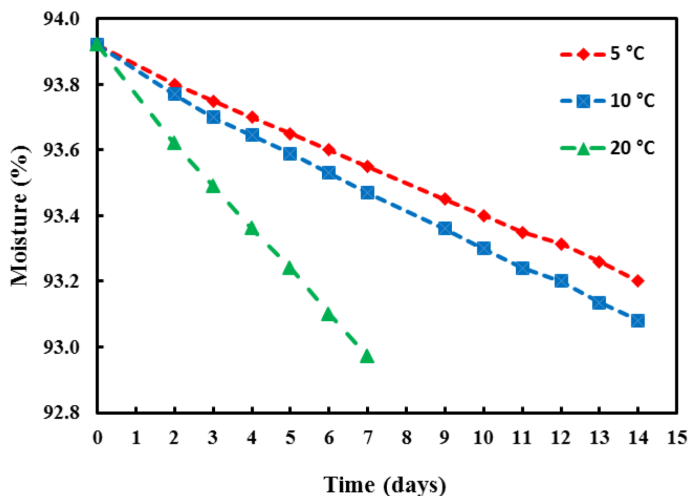

Figure 4.10. Moisture trend in corn salad stored at different temperautres.

Dehydration never exceed 1% of the starting weight no matter the storage temperature considered, although the largest process rate is observed at 20°C. A straight line fit of the data allows evaluation of the corresponding dehydration rate, namely, 0.0013, 0.0006, 0.0005 d^{-1}, at 20, 10 and 5°C, respectively.

4.5. CONCLUSIONS

The growth of the microbial population in fresh salad depends on the temperature and attains a similar steady plateau, no matter the temperature, although after different time lapses.
The steady microbial population attained has been used as a starting point for experiments of isothermal calorimetry performed at 30°C on salad samples stored for different time lapses at 5, 10 and 20°C. The increase of the microbial population (related to the imposed increase of temperature) was therefore a reliable biosensors for assessing the ageing deterioration of the product. The damage experienced by the product was found to be related to a combined effect of the storage duration and storage temperature. These results therefore showed that the calorimetric investigations can be of help in assessing the overall degradation of the fresh product in the framework of a TTT diagram, which implies the use a single deterioration parameter instead of a number of different parameters, each requiring an *ad hoc* interpretation.
Details of the present investigations will be reported in a ready to submit paper.

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APPENDIX 1. ABSTRACTS OF PAPERS, ORAL COMMUNICATIONS AND POSTERS

1. Pilu R, Landoni M, Cerino-Badone F, Haman N, Schiraldi A, Fessas D, Cesari V, Toschi I, Cremona R, Delogu C, Villa D, Cassani E, 2013, Low Phytic Acid 1 Mutation in Maize Modifies Density, Starch Properties, Cations, and Fiber Contents in the Seed. Journal of Agric Food Chem 61: 4622−4630.

2. Haman N, Corsini A, Signorelli M, Andreoni V, Schiraldi A, Fessas D, 2011, Isothermal calorimetry investigations of microbial growth in as contaminated media, 10th Mediterranean Conference on Calorimetry and Thermal Analysis, Porto, Portugal.

3. Haman N, Corsini A, Signorelli M, Andreoni V, Schiraldi A, Fessas D, 2012, Metabolism and growth of microorganisms in Arsenic-contaminated media Combining classical microbiology and Isothermal Calorimetry. XVII International Society for Biological Calorimetry Conference, Leipzig, Germany.

4. Haman N, 2011, Use of Isothermal Calorimetry to Monitor Microbial Growth in Food Systems and Soils. 16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 21-23 September, 2011 (Lodi, Italy).

5. Haman N, 2012, The microbial growth and Isothermal Calorimetry. 17th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 19-21 September, 2012 (Cesena, Italy).

6. Haman N, 2013, The microbial growth and Isothermal Calorimetry. 18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 25-27 September, 2013 (Conegliano, Italy)

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Low Phytic Acid 1 Mutation in Maize Modifies Density, Starch Properties, Cations, and Fiber Contents in the Seed

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ABSTRACT: Monogastric animals are unable to digest phytic acid, so it represents an antinutritional factor and also an environmental problem. One strategy to solve this problem is the utilization of low phytic acid (*lpa*) mutants that accumulate low levels of phytic P and high levels of free phosphate in the seeds; among the *lpa* maize mutants *lpa1* exhibited the highest reduction of phytic acid in the seed. This study indicated that the low phytic acid mutations exerted pleiotropic effects not directly connected to the phytic acid pathway, such as on seed density, content of ions, and the antioxidant compounds present in the kernels. Furthermore some nutritional properties of the flour were altered by the *lpa1* mutations, in particular lignin and protein content, while the starch does not seem to be modified as to the total amount and in the amylose/amylopectin ratio, but alterations were noticed in the structure and size of granules.

KEYWORDS: *maize, phytic acid, starch, ions, storage proteins, lignin content*

10th Mediterranean Conference on Calorimetry and Thermal Analysis, Porto, Portugal, 24-27 July 2011.

ISOTHERMAL CALORIMETRY INVESTIGATIONS OF MICROBIAL GROWTH IN As CONTAMINATED MEDIA

Nabil Haman, A. Corsini, M. Signorelli, V. Andreoni, A. Schiraldi, D. Fessas

Arsenic is a ubiquitous contaminant related to natural weathering and industrial or agricultural activities and strongly affect the quality of cultivable soils used for the production of edible crops.
Arsenic toxicity and mobility depends on chemical speciation and the oxidation state. For instance inorganic arsenite [As(III)] is more toxic and mobile than arsenate [As(V)].
Microorganisms can directly affect the redox balance As(V)/As(III) shifting it toward the species that can be easier processed and/or absorbed by scavenger plants.
Isothermal calorimetry represents a powerful method to monitor directly the biological activity of such living systems since provide direct quantitative information both on the energetic and kinetic behaviour.
In this study, isothermal calorimetry was applied to assess the effects of As on the growth, metabolism and the As(V)/As(III) redox capability of *Achromobacter* sp. As5-13 strain, a well characterized microorganism isolated from an agricultural soil. Complementary classical microbiological methods were also performed in parallel.

XVII International Society for Biological Calorimetry Conference, Leipzig, Germany, 03-06 June 2012.

Metabolism and growth of microorganisms in arsenic-contaminated media Combining classical microbiology and Isothermal Calorimetry

Nabil Haman, A. Corsini, M. Signorelli, V. Andreoni, A. Schiraldi, D. Fessas

Arsenic is a ubiquitous contaminant related to natural weathering and industrial or agricultural activities and strongly affect the quality of cultivable soils used for the production of edible crops.
The toxicity and mobility of this element depends on chemical speciation and its oxidation state and is specific for each microorganism.
Isothermal calorimetry (IC) and classical microbiological methods were applied to evaluate the influence of As (V) and As (III) on the growth and metabolism of Bacillus sp. 3.2 in different culture medium, namely, Tris Mineral Medium with Gluconate (TMMG) with the addition of yeast extract, and Tryptone Soya Broth (TSB).
The IC thermograms in combination with the microbiological counts and the chemical analysis data on the As oxidation status during the microbial activity, permitted to discriminate the influence of arsenic on the energetic aspects of the microbial metabolism in conditions that mimic different kind of cultivable soils.

16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 21-23 September, 2011 (Lodi, Italy)

Use of isothermal calorimetry to monitor microbial growth in food systems and soils

Nabil HAMAN

This PhD thesis research project is aimed to exploit Isothermal Calorimetry methods to asses the growth kinetic and metabolic activity of microorganisms involved in the food systems both on the agricultural production of the raw materials and the transformation processes.

17th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 19-21 September, 2012 (Cesena, Italy)

The microbial growth and Isothermal Calorimetry

Nabil Haman

Isothermal calorimetry, in this study was applied to assess microbial growth parameters as metabolic activity. Thus, calorimetric measurements are proven to be suitable to evaluate the toxic effect of arsenic on growth of different microbial strains. Other parallel measurements focused mainly on the toxic effect of arsenic with plenty of conventional methods to monitor microbial growth such as turbidity or determination of CFU. This technique was also used to assess the shelf life of fresh food products by monitoring the microbial growth.

18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 25-27 September, 2013 (Conegliano, Italy)

The microbial growth and isothermal calorimetry

Nabil Haman

Isothermal Calorimetry (IC) can be used to assess microbial growth and metabolic activity (Barros et al., 2007). In the present study, calorimetric measurements are proven to be suitable to evaluate the toxic effect of arsenic on the growth of different microbial strains isolated from soil and groundwater. Other parallel measurements focused mainly on the toxic effect of arsenic were performed with other conventional such as turbidity for the CFU count and standard chemical analysis to determine the arsenic concentration.
IC was also used to monitor the microbial spoilage of fresh food vegetables products and assess their shelf life in parallel with other Chemico-physical analysis (Riva *et al*., 2001).

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