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FORMULATION OF FUNCTIONAL FOODS USING COSTITUENTS MODIFIED BY BIOCHEMICAL AND PHYSICAL PROCESSES

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Chapter I

Preface

More recently the food industry, the agricultural community, and now consumers have shown a growing interest in the so-called functional foods. Such foods present a unique and difficult challenges to those in the food composition community and the many entities and individuals that depend on high-quality food composition data. Functional foods can arise from a desire to provide additional benefits to consumers in the way of enhanced nutrition. They can also be useful in making nutrients more available by providing particular dietary components in foods that will increase their availability and palatability beyond that which might normally be consumed (*Roberfroid M.B., 1999)*.

While functional foods can offer potential benefits, there are a number of concerns that need to be considered before we can change the food supply in beneficial ways. Foods contain a variety of nutrients and nutritionally beneficial components many of which are known, but many or which are not yet determined. Despite the fact that technology has progressed to a point where changes in foods can be made easily, one could reasonably argue that we do not know enough about the role of all dietary components, particularly the non-traditional or emerging nutrients such as phytonutrients, to make meaningful changes to the food supply *(No-Seong Kwak et al., 2001)*.

Another nutritional concern with functional foods is the possibility of consuming an excess of nutrients, some of which may have toxic properties. While a functional foods approach might be desirable to increase the intake of particular nutrients, one of the concerns related to functional foods could be that by enhancing nutrient content, we may be reducing the variety in the diet as consumers rely on a smaller number of foods

to meet their dietary needs. Most would agree that variety in the diet is essential, and functional foods can have the undesirable effect of reducing variety.

The concept of functional foods (*Farr D.R., 1997)* is about:

- Contribution to the improvement of the diet and the maintenance and enhancement of health should be expected.
- The health benefit of the food or relevant components should have a medical and/or nutritional basis.
- The appropriate level of consumption should be definable for the food or relevant components based on medical or nutritional knowledge.
- The food or relevant components should be safe, based on appropriate data.
- The relevant components should be well defined in terms of physiochemical properties and by methods of qualitative and quantitative determination.
- The composition of the product should not be notably defective in comparison with the composition of nutritive components that are normally contained in similar types of foods.
- The product should be a food consumed in ordinary dietary patterns, rather than those consumed only occasionally.
- The product should be in the form of ordinary foods.
- The food are relevant components should not be those exclusively used as medical drugs.

The variety of functional foods *(Spence J.T., 2006)* can be indicated as:

- *Fortified foods*

  The simplest types of functional foods are those products that are fortified with additional nutrients. This approach of fortification has proven to be an effective and economical way to improve nutrient quality and provide benefits to consumers. However, one can easily see the problems that it presents with regard to food composition. A problem can exist in that the amount of fortification may not be accurate, and that fortification of identical products at differing manufacturing plants can lead to the same product with differing amounts of the nutrient that has been used to fortify the product.

- *Enriched foods*

Other examples of functional foods are enriched products that might add additional components or components not normally found in great quantity in a particular food. This type of products provide clear benefits in dealing with a problem nutrient such as calcium, or have been shown to be effective in promoting cardiovascular health, respectively. However, with regard to food composition, this can create problems. The growing dissimilarity of what only a few years ago was a single product adds a higher degree of complexity in tracking food composition data.

Another interesting area of functional foods and one that has reached the grocery shelf is that of components classified as probiotics and prebiotics. Probiotics are live microbial food ingredients that have a beneficial effect on human health *(Sanders M.E., 1998)*; they are traditionally found in fermented dairy products and fermented vegetables *(Knorr D., 1998; Caplice E. et al., 1999; Nout M.J.R. and Motarjemi Y., 1997; Nout M.J.R. and Ngoddy P.O., 1997)*. Prebiotics are typically fermentable dietary fibres that provide a gastrointestinal environment in which beneficial bacteria can thrive *(Huebner J. et al., 2007)*. Moreover, prebioticity can be provided by death microorganisms that offer similar effect of fermentable fibres (*Ouwehand A.C. and Salminen S.J., 1998)*.

- *Altered products*

Using different ingredients, food products can be developed whereby some potentially harmful or undesiderable constituents could be replaced by more beneficial components, ideally without affecting product quality.

- *Enhanced commodities*

An exciting class of functional foods in enhanced commodities. Plant breeders can develop amazing varieties of products that have potentially important benefits to consumers. Examples include high lysine corn, fruits and vegetables with enhanced content of vitamins, and overproduction of phytonutrients in a

variety of fruits and vegetables including the insertion of some of those components into food plants that do not normally produce those dietary components, such as golden rice or carotenoid containing potatoes.

The aim of the present work is to develop dry constituent of foods having probiotic and/or prebiotic effects.

These products are obtained by using lactic fermentation of foods substrates, as cereals suspensions or skim milk, followed by a suitable drying process. The drying treatment is order to increase the shelf life of the constituents, leaving as long as possible the probiotic/prebiotic activities.

Probiotic concern is linked to residual viable microorganisms that surviving to heat and drying injuries, while prebiotic effects are linked to non-viable bacteria structures that colonizing the bowel mucus avoid the growth of undesired noxious bacteria.

Recent in-vivo tests using cavie, have shown a considerable increase of the immunological response of the hosts.

Drying functional foods with pro/prebiotic properties, are obtained by means the fermentation of cereal suspension and of skim milk. After fermentation product are dried using a suitable process in order to leave unaltered nutritional properties and health characteristics of functional food.

In first case, application of cereals and cereal components in functional foods for infant and children (*Haschke F. et al., 2001)* offer an alternative for the production of functional foods. Cereal grains are considered to be one of the most important sources of dietary proteins, carbohydrates, vitamins, minerals and fibre for people all over the world (*Blandino A. et al., 2003)*. However, the nutritional quality of cereals and the sensorial properties of their products are sometimes inferior or poor in comparison with milk and milk products (*Kedia G. et al., 2007)*. The reasons behind this are the lower protein content, the deficiency of certain essential amino acids (lysine), the low starch availability, the presence of determined antinutrients (phytic acid, tannins and polyphenols) and the coarse nature of the grains (*Wronkowska M. et al., 2006)*.

Cereals are used for sourdough production, a fermentation process that nowadays is performed by using defined mixed starter cultures, consisting of lactic acid bacteria

strains isolated from naturals environments. Other cereal-based fermented foods are produced indigenously in Asia and Africa by natural lactic acid fermentation under uncontrolled conditions. However, as natural fermentations rely on microbial populations present in the raw material, these products exhibit substantial variations in flavour and quality. The good adaptation of lactic acid bacteria in cereals suggests that the utilization of a potentially probiotic strain as starter culture in a cereal substrate would produce a fermented food with defined and consistent characteristics and possibly health-promoting properties. However, several technological aspects have to be considered in the design of such a novel food fermentation process, such as the composition and processing of the raw material, the growth capacity and productivity of the starter culture and the stability of the final product during storage.

The functional foods based on cereals is a challenging perspective for the development of new technologies of cereal processing that enhance their health potential and the acceptability of the food product are the primary importance.

In the last case, the reasons for fermenting milk are numerous and, although the primary function is to extend its shelf-life, other advantages, such as improving the taste of milk, enhancing the digestibility of the product and the manufacture of a wide range of product (i.e. from yoghurt to concentrated yoghurt to cheese) should not be overlooked. Originally, the souring of milk was by no means uniform, and fermentations brought about by mixtures of lactic and non-lactic acid bacteria gave rise to products that were insipid and stable. Furthermore, the coagula were often irregular, filled with gas holes and showed signs of whey syneresis. By contrast, pure cultures of lactic acid bacteria act on milk to produce a fermentate that is pleasant to eat or drink and, over the years, communities in different countries derived fermentation processes that brought the 'souring of milk' under control.

For the development of dairy-based functional foods containing high numbers of viable probiotics, the culture should have the ability to grow in substrates, remain viable and retain probiotic properties during production and storage (shelf-life) of the probiotic food product. The harsh conditions of the gut, including the acid nature of the stomach and the presence of bile in the intestine, can adversely affect the viability of probiotic cultures following consumption.

After fermentation process for both cereals and milk as suitable drying process can be selected spray-drying. This dry process is able to produce large amounts of dried ingredients relatively inexpensively, the spray-dried powders can be transported at a low cost and can be stored in a stable form for prolonged periods. Spray-dried powders harbouring relatives high levels of viable microorganisms provide a convenient form of these cultures for storage purposes and applications in functional food developments. However, a major limitation of spray-drying is about probiotic cultures for the loss of viability which occurs during processing and storage of the powders. The survival rate of the culture during spray drying and subsequent storage depends upon a number of factors, including the species and strain of culture, the drying conditions, the inoculum and medium used, preadaptation of the culture to acquire resistance to processing conditions and the use of protective agents.

However, it is obvious that the exposure to high air temperatures, which are required to facilitate water evaporation during the passage of the bacteria in the spray drying chamber, exerts a negative impact on their viability and hence their activity in the spray-dried product. Furthermore, since water contributes to the stability of biological molecules, the removal of water may cause irreversible changes in the structural and functional integrity of bacterial membranes and proteins. Preservation of these essential functions and structure is crucial for the survival of bacteria and the retention of their functionality.

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The probiotic survival rate was inversely proportional to outlet temperatures and the residual moisture content increased as the air outlet temperature was reduced. Thus, a compromise in terms of selection of air outlet temperature is required for optimal drying. In particular the survival rates for bacteria during spray drying ranged from 97% at an outlet temperature of 70 to 75°C to 0% at 120°C *(Gardiner G.E. et al., 2000).*

Possible reasons for loss of viability occurring during heating can now be placed in a temperature sequence as follows: below 64°C critical sites are structures contained in or making up the cell membrane. For temperature of 65°C and immediately above, ribosomes and/or proteins denaturation as well as cell wall damage may be responsible

for thermal death. Ribosome denaturation occurs in the same temperature region as thermal inactivation. It was suggested that half the enthalpy of ribosome denaturation is associated with protein denaturation and/or disruption of higher order interactions. Heat denaturation/melting of DNA may be responsible for death at temperatures near 90°C and above (*Teixeira et al., 1997*).

Main activities of the present work are detailed in the following chapters:

1. *State-of art of the production of probiotic/prebiotic foods.*
2. *Selection of bacterial strain.*
3. *Analysis of fermentation of substrate on a lab-scale.*
4. *Development of a fermentation reactor on a pilot-scale.*
5. *Analysis of fermentation of substrate on a pilot-scale.*
6. *Drying of a fermented product on a pilot scale.*
7. *Development of a fermentative reactor on industrial scale.*

Chapter II

State – of – art of the production of probiotic/prebiotic foods

The food industry is directing new product development towards the area of functional foods and functional foods ingredients due to consumers' demand for healthier foods. There is a clear relationship between the food we eat and our health. But beyond supplying a source of calories and nutrients to prevent nutritional diseases such as malnutrition, scurvy and rickets, it is clear that the food we eat can contribute in less overt ways to maintenance of health and prevention of a broad range of diseases.

The human gastrointestinal tract is a kinetic microecosystem that enables normal physiological functions of the host organism unless harmful and potentially pathogenic bacteria dominate it. Maintaining a proper equilibrium of the microflora may be ensured by systematic supplementation of the diet with probiotics, prebiotics or synbiotics.

The main importance is known the value of functional foods, in particular the properties of probiotics and prebiotics foods to health, and study a fermentation processes that is able to create this functional foods from substrate of interest.

Fundamental to start with experimental analyses is identified the type of fermentation, the type of fermentator, an optimal drying method and identify parameters that are on the bioprocess base.

2.1 Functional food: probiotics and prebiotics.

The word *probiotic* is derived from two Greek words meaning 'for life'. Early attempts to use the term to mean a microbial substance which stimulates the growth of another microorganisms *(Lilley & Stillwell, 1965)* or tissue extracts which improved microbial growth *(Sperti, 1971)* did not gain general acceptance.

Probiotics are living microorganisms that when ingested in certain numbers exert a beneficial effect beyond that of their nutrition value. The exact mechanism of action of probiotics is still unclear. Initial theories revolved around "competitive exclusion," in which "good" bacteria eliminated or suppressed "bad" bacteria. Although competitive exclusion may play a role in probiotic therapy, it is unlikely to account for the wide array of beneficial effects that have been reported in other species. Health advantages associated with probiotic intake are: alleviation of symptoms of lactose malabsorption, increase in natural resistance to infectious diseases of the intestinal tract, suppression of cancer, reduction in serum cholesterol concentrations, improved digestion, and stimulation of gastrointestinal immunity *(Collins & Gibson, 1999)*, although, well-characterized strains with proven clinical effects are not numerous. Bacteria belonging to the genera *Bifidobacterium* and *Lactobacillus* are most often used as probiotic supplements for food. They exert only beneficial properties with regard to human health, such as inhibition of growth of exogenous and/or harmful bacteria, stimulation of immune functions, anti-tumor properties, cholesterol reduction, aid in digestion and/or absorption of food ingredients/minerals and synthesis of vitamins *(Gibson, 1998)*. The other commensal microflora additionally produces toxins, potential carcinogens, toxic H_2S and/or causes intestinal putrefaction, whereas activity of pathogenic bacteria results in diarrhea or constipation, infections and harmful systemic effects. A number of organisms have been demonstrated to possess beneficial probiotic properties, great variation exists within species of microorganisms. Beneficial effects cannot be extrapolated between members of the same species, let alone the same genus. Individual bacterial or yeast strains must be tested to determine whether they possess beneficial properties. These include surviving passage through the stomach and small intestine, remaining viable during processing and storage, being nonpathogenic, and

exerting one or more beneficial effects such as antimicrobial factor production, immune regulation, or decreasing inflammation. In the absence of strain-specific in vitro and in vivo testing, determination of whether a certain strain could be beneficial is impossible.

A *prebiotic* is a nondigestible food ingredient that beneficially affects the host by stimulating growth and/or activity of certain bacterial components of the intestinal microflora. Prebiotics are neither hydrolyzed nor absorbed in the small intestine, are a selective substrate for potentially beneficial organisms in the colon, and alter the colonic microflora in a manner that produces beneficial effects. A number of food ingredients could act as prebiotics; however, the most commonly evaluated prebiotics are those that stimulate the lactic acid bacteria (lactobacilli, bifidobacteria, and enterococci) component of the microflora. These consist mainly of nondigestible fructooligosaccharides and soybean oligosaccharides.

Prebiotics may be administrated as daily or intermittent supplements or incorporated into commercial diets. An advantage of prebiotic therapy over probiotic therapy is that viable microorganisms are not required. Viability of prebiotics should not be lost during standard processing and storage, as opposed to the situation with probiotics.

Mechanisms by which probiotic and prebiotic supplements affect the microecology of the intestinal tract are non well studied, but at least four mechanisms of action have been observed:

1) Antibacterial agents (bacteriocins) that are produced and secreted by probiotic organisms may have an inhibitory effect on controlling pathogenic microflora;
2) Human breast milk may alter bacterial antagonism for essential nutrients and impede overgrowth of aerobes;
3) Stimulation of immune responses (e.g. antibody titers, macrophage activity, T cells, and interferon) may suppress potential pathogens;
4) Specific competition for adhesion receptors to gut epithelium may allow lactic acid bacteria and *Bifidobacteria* to occupy the niche normally required by toxin-producing organisms for colonization.

The various fermenting substrates for specific lactic acid bacteria and *Bifidobacteria* differ in their metabolic potential but in general include the following:

- Production of lactic acid and lesser amounts of acetic and formic acids;

- Production of antimicrobial bacterocins and fatty acid;
- Reduction in toxin-producing organisms and bacterial translocation.

The mechanisms behind specific benefits include:

- Strengthening of the gut mucosal barrier;
- Gut microflora modification;
- Adherence to intestinal mucosa with an ability to prevent adherence of pathogen proliferation;
- Modification of dietary proteins by the intestinal microflora;
- Modification of bacterial enzyme activity;
- Influence on gut mucosal permeability.

Substrates used in the production of functional foods are cereals and milk. A detailed description is show in the following.

2.2 Cereals in functional foods.

Cereals offer another alternative for production of functional foods *(Charalampopoulos D. et al., 2002)*. The multiple beneficial effects of cereals can be exploited in different ways leading to the design of novel cereal foods or cereal ingredients that can target specific populations. Cereals can be used as fermentable substrates for the growth of probiotic microorganisms. The main parameters that have to be considered are the composition and processing of the cereal grains, the substrate formulation, the growth capability and productivity of the starter culture, the stability of the probiotic strain during storage, the organoleptic properties and the nutritional value of the final product. Additionally, cereals can be used as sources of nondigestible carbohydrates that besides promoting several beneficial physiological effects can also selectively stimulate the growth of lactobacilli and bifidobacteria present in the colon and act as prebiotics. Cereals contain water-soluble fiber, such as α-glucan and arabinoxylan, oligosaccharides, such as galacto- and fructo-oligosaccharides and resistant starch, which have been suggested to fulfill the probiotic concept. Separation of specific fractions of fiber from different cereal varieties or cereal by-products, according to the

knowledge of fiber distribution in cereal grains, could be achieved through processing technologies, such as milling, sieving, and debranning or pearling. Finally, cereal constituents, such as starch, can be used as encapsulation materials for probiotics in order to improve their stability during storage and enhance their viability during their passage through the adverse conditions of the gastrointestinal tract. It could be concluded that functional foods based on cereals is a challenging perspective, however, the development of new technologies of cereal processing that enhance their health potential and the acceptability of the food product are of primary importance.

2.3 Milk in functional foods.

The health benefits of milk and fermented milk products have been known since medieval times. Drinking milk has taken the advantage of the extensive nutritional value not only to the child, but also to the adult and the elderly. The health benefits of dairy products are the result of biologically active components that are present in native milk and also, due to their suitably modulated activities produced through the action of probiotic bacteria, in the fermented or sour milk products. In addition to modification of several milk components the probiotics may act also directly as preventive agents, or in therapy of some contagious, atopic, tumor or other severe diseases (*Ferencìk and Ebringer, 2003; Gill and Guarner, 2004; Santosa et al., 2006).* The health-promoting effect of the prefermented functional foodstuffs materializes directly through interaction with consumed microorganisms (probiotic effect) or, indirectly, as a result of action of microbial metabolites generated during the fermentation process (biogenic effect). The most important biogenic metabolites include vitamins, proteins, peptides, oligosaccharides, and organic acids, including FAs.

In many cases, the health-promoting mechanisms of probiotic action are not sufficiently known. However, the majority of them are based on the positive effect they exert on the immune response, i.e. on their immunomodulatory activity (*Isolauri et al., 2001; Biancone et al., 2002).* In most cases, this is due to stimulation of natural immunity (*Newburg, 2005; Galdeano and Perdigon, 2006).* In doing so they modulate primarily

the production of cytokines and antimicrobial peptides (*Trebichavsky and Splichal, 2006).* This is the mode of action of not just typical sour milk functional foodstuffs, such as sour milk, kefir or yoghurt (*Meydani and Ha, 2000; Farnworth, 2005)* produced by the food-processing industry, but also that of the diet supplements containing the probiotic bacteria in pure form. However, the latter are the products of pharmaceutical industry and, in contrast to functional foodstuffs, they have a standard composition, and known immunomodulatory characteristics, verified both experimentally and in controlled clinical studies. In terms of their quality and efficiently they are also under regular pharmaceutical control. It will be therefore more precise to call them **immunobiotics**, in order to distinguish them from classical probiotics in functional foodstuffs (*Clancy, 2003).*

However, one major part of milk-whey, has traditionally not been paid as much attention as happened with sour milk, because it is a by-product of cheese making. During the Middle Age whey was considered not only as a medicine but also as a kind balm and an aphrodisiac. Whey proteins, namely α-lactalbumin, β-lactoglobulin, lactoferrin, lactoperoxidase and serum albumin, possess important nutritional and biological properties particularly with regard to prevention of diseases. Antimicrobial, anticarcinogenic, immunostimulatory and other health-promoting activities of whey proteins. Dairy products and their components, mainly whey, contribute to the regulation of body mass by providing satiety signals. Therefore, whey proteins have a potential as physiologically dairy components for a person with obesity and metabolic syndrome. High-protein dairy products and, in particular those that contain whey proteins, may reduce fat deposition and improve insulin sensitivity (*Luhovyy et al., 2007; Dunshea et al., 2007).* Dairy proteins and peptides also improve the bioavailability of minerals and trace elements, such as calcium, magnesium, manganese, zinc, selenium and iron (*Vegarud et al.,2000).*

2.4 Production of functional food: fermentation.

Using its most rigorous, chemical definition the term 'fermentation' is applied to describe a strictly anaerobic process; however, the general understanding of the term now encompasses both aerobic and anaerobic carbohydrate breakdown processes.
Fermentation is one such important process which significantly lowers the content of antinutrients *(Sharma and Kapoor, 1996; Sripriya et al., 1997)* and thereby improves the nutritive value of food grains. Fermentation is a process to improve the digestibility, quality, safety and physico-chemical properties of the raw material and which is aimed to produce probiotics and functional foods or food ingredients can be counterproductive to the viability of microorganisms because it requires maximum productivity of microorganisms. This can lead to poor microbial viabilities in the fermented product. Consequently, the challenge is to retain and optimize microbial viability at the same time as improving productivity.

2.4.1 Fermentation equipment.

Any large-scale operation involving the transformation of some raw material (biological or non-biological) into some product by means of microorganisms, animal or plant cell cultures, or by material (e.g. enzymes, organelles) derived from them, may be termed a "bioprocess". The raw material or feedstock must be converted to a form which is suitable for processing. This is done in a pretreatment step which may involve one or more of the operations shown in Figure 2.1.

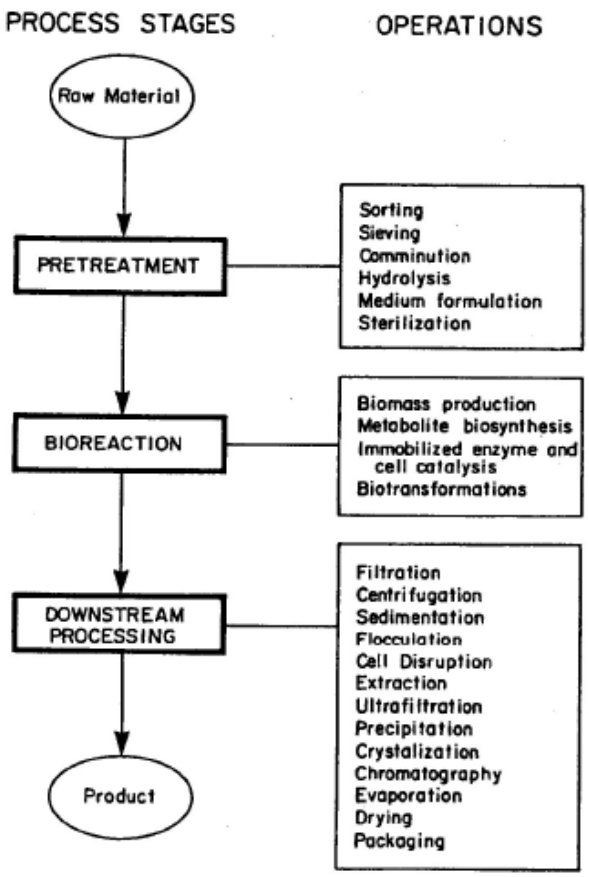


Fig.2.1: Bioprocess stages and the commonly used operations in them (Y. Chisti and Murray Moo-Young, 1999).

Frequently, the well established chemical engineering operations suffice for the pretreatment stage and these will not be discussed further.

The pretreatment step is followed by one or more bioreaction stages where the desired biotransformation takes place. The transformation may involve the conversion of a substrate to biomass or biomass and some biochemical or enzyme. Alternatively, the conversion may use dead whole cells (immobilized or in suspension) or enzyme as the biocatalytic agency.

Bioreactors form the core of the bioreaction step. The material produced in the bioreactors must usually be processed further in the downstream section of the process

to convert it to a useful form. Downstream processing consists of predominantly physical operations which are aimed at concentration and purification of the product. The purified product may have to be in different physical forms (liquid, slurry, powder, crystalline) for different applications.

The properties of biological materials impose significant unique constraints on the bioreaction and downstream processing stage.

Industrial fermentations may be carried out either batchwise, as fed-batch operations, or as continuous cultures (Fig. 2.2).

Batch and fed-batch operations are quite common, continuous fermentations being relatively rare.

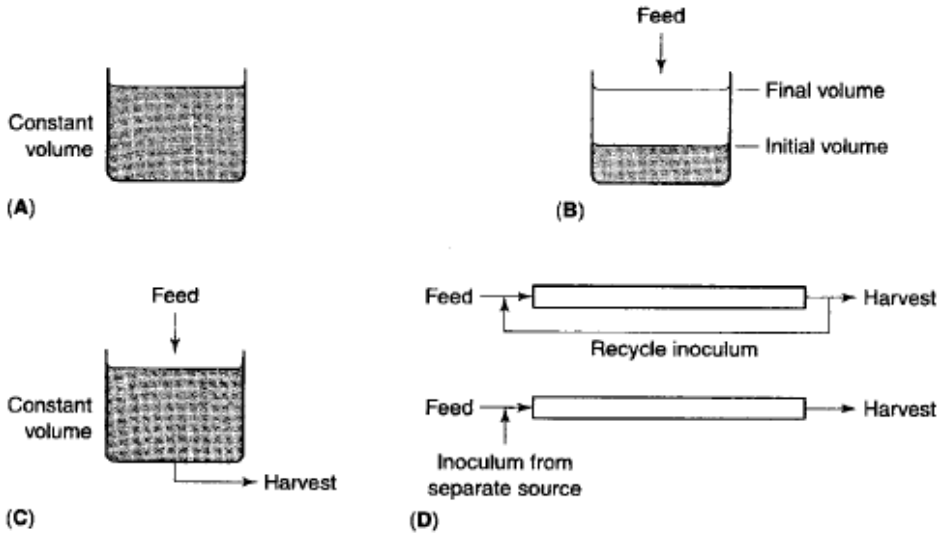


Figure 2.2: Fermentation methodologies. (A) Batch fermentation. (B) Fed-batch culture. (C) Continuous-flow well-mixed fermentation. (D) Continuous plug flow fermentation, with and without recycling of inoculum.(Y. Chisti, 1999).

In batch processing, a batch of culture medium in a fermenter is inoculated with a microorganism (the 'starter culture'). The fermentation proceeds for a certain duration (the 'fermentation time' or 'batch time'), and the product is harvested. Batch fermentations typically extend over 4-5 days, but some traditional food fermentations may last months.

In fed-batch fermentations, sterile culture medium is added either continuously or periodically to the inoculated fermentation batch. The volume of the fermenting broth increases with each addition of the medium, and the fermenter is harvested after the batch time.

In continuous fermentations, sterile medium is fed continuously into a fermenter and the fermented product is continuously withdrawn, so the fermentations are started as batch cultures and feeding begins after the microbial population has reached a certain concentration. In some continuous fermentations, a small part of the harvested culture may be recycled, to continuously inoculate the sterile feed medium entering the fermenter (Fig. 2.2(D)).

Whether continuous inoculation is necessary depends on the type of mixing in the fermenter. 'Plug flow' fermentation devices (Fig. 2.2(D)), such as long tubes that do not allow back mixing, must be inoculated continuously. Elements of fluid moving along in a plug flow device behave like tiny batch fermenters. Hence, true batch fermentation processes are relatively easily transformed into continuous operations in plug flow fermenters, especially if pH control and aeration are not required. Continuous cultures are particularly susceptible to microbial contamination, but in some cases the fermentation conditions may be selected (e.g. low pH, high alcohol or salt content) to favour the desired microorganisms compared to potential contaminants.

In a 'well-mixed' continuous fermenter (Fig. 2.2(C)), the feed rate of the medium should be such that the dilution rate, i.e. the ratio of the volumetric feed rate to the constant culture volume, remains less than the maximum specific growth rate of the microorganism in the particular medium and at the particular fermentation conditions. If the dilution rate exceeds the maximum specific growth rate, the microorganism will be washed out of the fermenter.

Industrial fermentations are mostly batch operations. Typically, a pure starter culture (or seed), maintained under carefully controlled conditions, is used to inoculate sterile Petri dishes or liquid medium in the shake flasks. After sufficient growth, the pre-culture is used to inoculate the 'seed' fermenter. Because industrial fermentations tend to be large (typically 150-250 m^3), the inoculum is built up through several successively larger stages, to 5-10% of the working volume of the production fermenter. A culture in rapid

exponential growth is normally used for inoculation. Slower-growing microorganisms require larger inocula, to reduce the total duration of the fermentation. An excessively long fermentation time (or batch time) reduces productivity (amount of product produced per unit time per unit volume of fermenter), and increases costs. Sometimes inoculation spores, produced as seeds, are blown directly into large fermentation vessels with the ingoing air.

2.5 Batch fermentation.

Batch fermentation is the simplest mode of operation, and is often used in the laboratory to obtain substantial quantities of cells or product for further analysis. A batch fermentation is a closed system, where all of the nutrients required for the organisms' growth and product formation are contained within the vessel at the start of the fermentation process. The vessel can take the form of a shake flask, single use disposable system, or, for tighter control of parameters such as oxygen transfer, pH, agitation, etc., a bioreactor can be used (Figure 2.3). Historically, these processes would have involved nonsterile systems with self-selecting or natural inoculants. However, nowadays nearly all fermentation processes involve inoculation of a selected and specially bred strain of microbe, plant or animal cell into a sterile medium held within a sterile fermenter vessel. After medium sterilization, the organism is inoculated into the vessel and allowed to grow. The fermentation is terminated when one or more of the following has been reached: (i) microbial growth has stopped due to the depletion of the nutrients or the build of toxic compounds; (ii) after a fixed predetermined period of time; (iii) the concentration of desired product has been achieved.

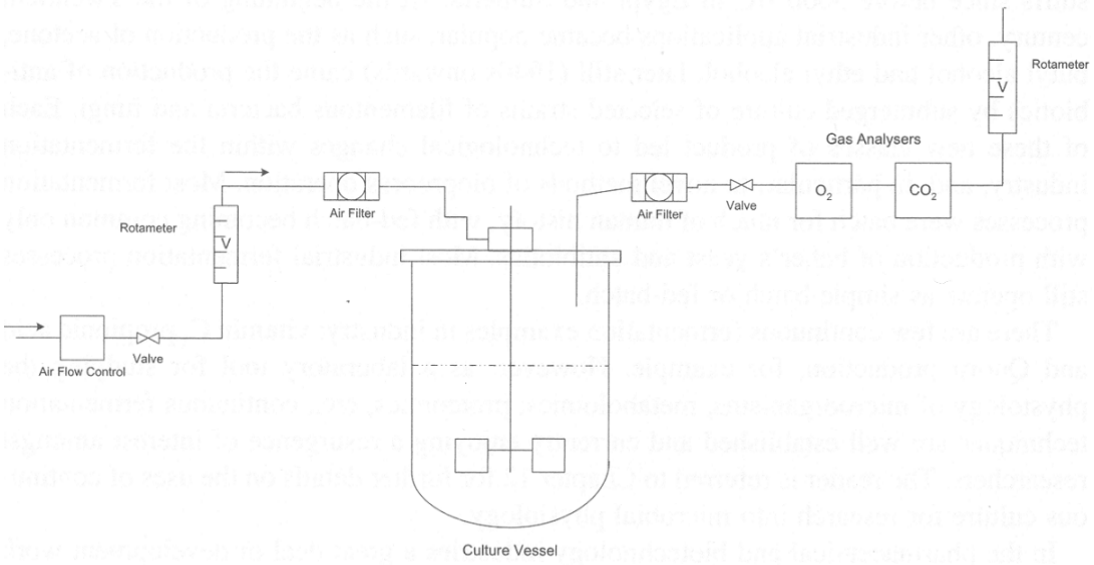


Fig.2.3: A diagram of a simple batch fermentation. The system is 'closed', containing all the nutrients required by the organism prior to inoculation, except for the gaseous requirement, which is continuously added to, and removed from, the reactor via sterilizing-grade hydrophobic filters (Brian McNeil et al., 2008).

It is difficult to obtain useful kinetic information from reactors that have spatially nonuniform conditions. Hence it desirable to study kinetics in reactors that are well mixed (Figure 2.4).

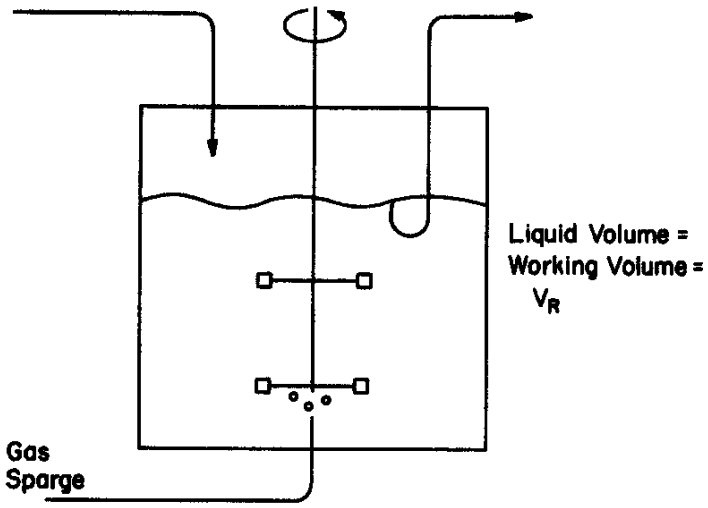


Fig.2.4: Well mixed reactor.

Many biochemical processes involve batch growth of cell population. After *seeding* in a liquid *medium* with an *inoculum* of living cells, nothing (except possibly some gas) is added to the *culture* or removed from it as growth proceeds. Typically in such a reactor, the concentration of nutrients, cells, and products vary with time as growth proceeds.

Batch growth refers to culturing cells in a vessel with an initial charge of medium that is not altered by further nutrient addition or removal.

The quantification of cell concentration in a culture medium is essential for the determination of the kinetics and stoichiometry of microbial growth. The methods used in the quantification of cell concentration can be classified in two categories: direct and indirect. In many cases, the direct methods are not feasible due to the presence of suspended solids or other interfering compounds in the medium. Either cell number or cell mass can be quantified depending on the type of information needed and the properties of the system. Cell mass concentration is often preferred to the measurement of cell number density when only one is measured, but the combination of two measurements is often desirable.

2.5.1 Growth patterns and kinetics in batch culture.

When a liquid nutrient medium is inoculated with a seed culture, the organisms selectively take up dissolved nutrients from the medium and convert them into biomass. A typical batch growth curve includes the following phases:

1) Lag phase;
2) Logarithmic or exponential growth phase;
3) Deceleration phase;
4) Stationary phase;
5) Death phase.

Figure 2.5 describes a batch growth cycle.

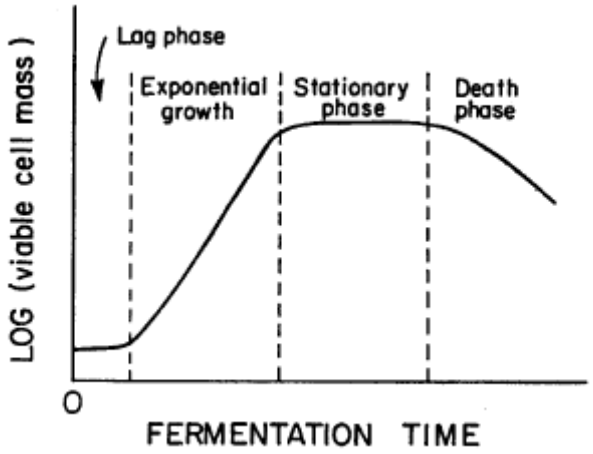


Fig.2.5: Batch growth cycle (Y. Chisti and Murray Moo-Young, 1999).

The *lag phase* occurs immediately after inoculation and is a period of adaptation of cells to a new environment. Microorganisms reorganize their molecular constituents when they are transferred to a new medium. Depending on the composition of nutrients, new enzymes are synthesized, the synthesis of some other enzymes is repressed, and the internal machinery of cells is adapted to the new environmental conditions. During this phase, cell mass may increase a little, without an increase in cell number density. When the inoculum is small and has a low fraction of cells that are viable, there may be a pseudo lag phase, which is a result, not of adaptation, but of small inoculum size or poor

condition of the inoculum. Low concentration of some nutrients and growth factors may also cause a long lag phase. The age of the inoculum culture has a strong effect on the length of lag phase. The age refers to how long a culture has been maintained in a batch culture. Usually, the lag period increases with the age of the inoculum. To minimize the duration of the lag phase, cells should be adapted to the growth medium and conditions before inoculation, and cells should be young (or exponential phase cells) and active, and the inoculum size should be large (5% to 10% by volume).

The *exponential growth phase* is also known as the *logarithmic growth phase*. In this phase, the cells have adjusted to their new environment. After this adaptation period, cells can multiply rapidly, and cell mass and cell number density increase exponentially with time. This is a period of *balanced growth*, in which all components of a cell grow at the same rate. That is, the average composition of a single cell remains approximately constant during this phase of growth. During balanced growth, the net specific growth rate determined from either cell number or cell mass would be the same. Since the nutrient concentrations are large in this phase, the growth rate is independent of nutrient concentration. The exponential growth rate is first order:

$$\frac{dX}{dt} = \mu_{net} X, \quad X = X_0 \quad \text{at} \quad t = 0 \tag{1}$$

integration of eq. 1 yields

$$\ln \frac{X}{X_0} = \mu_{net} t, \quad \text{or} \quad X = X_0 e^{\mu_{net} t} \tag{2}$$

where X and X_0 are cell concentration at time t and t = 0.

The *deceleration growth phase* follows the exponential phase. In this phase, growth decelerates due to either depletion of one or more essential nutrients or the accumulation of toxic by-product of growth. For a typical bacterial culture, these changes occur over a very short period of time. The rapidly changing environment

results in *unbalanced growth.* In the exponential phase, the cellular metabolic control system is set to achieve maximum rates of reproduction. In the deceleration phase, the stresses induced by nutrient depletion or waste accumulation cause a restructuring of the cell to increase the prospects of cellular survival in a hostile environment.

The *stationary phase* starts at the end of the deceleration phase, when the net growth rate is zero (no cell division) or when the growth rate is equal to the death rate. Even thought the net growth rate is zero during the stationary phase, cells are still metabolically active and produce secondary metabolites. *Primary metabolites* are growth-related products and *secondary metabolites* are nongrowth-related. In fact, the production of certain metabolites in enhanced during the stationary phase (e.g. antibiotics, some hormones) due to metabolite deregulation. During the course of the stationary phase, one or more of the following phenomena may take place:

- Total cell mass concentration may stay constant, but the number of viable cells may decrease.
- Cell lysis may occur and viable cell mass may drop. A second growth phase may occur and cells may grow on lysis products of lysed cells (cryptic growth).
- Cells may not be growing but may have active metabolism to produce secondary metabolites. Cellular regulation changes when concentrations of certain metabolites (carbon, nitrogen, phosphate) are low. Secondary metabolites are produced as a result of metabolite deregulation.

The reason for termination of growth may be either exhaustion of an essential nutrient or accumulation of toxic products. If an inhibitory product is produced and accumulates in the medium, the growth rate will slow down, depending on inhibitor production, and at a certain level of inhibitor concentration, growth will stop.

The *death phase* (or decline phase) follows the stationary phase. However, some cell death may start during the stationary phase, and a clear demarcation between these two phases is not always possible. At the end of stationary phase, because of either nutrient depletion or toxic product accumulation, the death phase begins.

2.5.2 Quantifying growth kinetics.

Cellular composition and biosynthetic capabilities change in response to new growth conditions (*unbalanced growth)*, although a constant cellular composition and balanced growth can predominate in the exponential growth phase. If the decelerating growth phase is due to substrate depletion rather than inhibition by toxins, the growth rate decreases in relation to decreasing substrate concentrations. In the stationary and death phases, the distribution of properties among individuals is important (e.g. cryptic death). Although these kinetic ideas are evident in batch culture, they are equally evident and important in other modes of culture (e.g. continuous culture).

The relationship of specific growth rate to substrate concentration often assumes the form of saturation kinetics. Here it is assume that a single chemical species, S, is growth-rate limiting (i.e., an increase in S influences growth rate, while changes in other nutrient concentrations have no effect). These kinetics are similar to the Langmuir-Hinshelwood (or Hougen-Watson) kinetics in traditional chemical kinetics or Michelis-Menten kinetics for enzyme reactions. When applied to cellular systems, these kinetics can be described by the *Monod equation*:

$$\mu_g = \frac{\mu_m S}{K_s + S} \qquad (3)$$

Where μ_m is the maximum specific growth rate when $S >> K_s$. If endogenous metabolism is unimportant, the $\mu_{net} = \mu_g$. The constant K_s is known as the *saturation constant or half-velocity constant* and is equal to the concentration of the rate-limiting substrate when the specific rate of growth is equal to one-half of the maximum. That is, $K_s = S$ when $\mu_g = 0.5\ \mu_{max}$. In general, $\mu_g = \mu_m$ for $S >> K_s$ and $\mu_g = (\mu_m / K_s)S$ for $S << K_s$. The Monod equation is semiempirical; it derives from the premise that a single enzyme system with Michaelis-Menten kinetics is responsible for intake of S, and the amount of that enzyme or its catalytic activity is sufficiently low to be growth-rate limiting. This simple premise is rarely, if ever, true; however, the Monod equation empirically fits a

wide range of data satisfactorily and is the most commonly applied unstructured, nonsegregated model of microbial growth.

The Monod equation describes substrate-limited growth only when growth is slow and population density is low. Under this circumstances, environmental conditions can be related simply to S. If the consumption of a carbon-energy substrate is rapid, then the release of toxic waste products is more likely (due to energy-spilling reactions). At high population levels, the build-up of toxic metabolic by-products becomes more important. The following rate expressions have been proposed for rapidly growing dense cultures:

$$\mu_g = \frac{\mu_m S}{K_{s0} S_0 + S} \tag{4}$$

or

$$\mu_g = \frac{\mu_m S}{K_{s1} + K_{s0} S_0 + S} \tag{5}$$

where S_0 is the initial concentration of the substrate and K_{s0} is dimensionless.

2.5.3 Advantages of Batch Culture.

- Simplicity of use. A batch culture can be easily readied, and, depending on the microorganism used, can be finished in less than 24 hours.
- Operability and reliability: less likely to have instrument failure on short batch runs;
- Production of secondary metabolites that are not growth-related (i.e., produced when the organism enters stationary phase);
- Fewer possibilities of contamination: all of the materials required for the bioprocess are present in the vessel and sterilized before the run starts. The only material added (with the exception of the inoculum at the beginning of the bioprocess) and removed during the course of a batch fermentation are the gas exchange, and if using a

bioreactor, sterile antifoam and pH control solutions if required.

- It is easy to assign a unique batch number to each run, generating high confidence in the history of each batch of product. This is critically important in a highly regulated environment.

2.5.4 Disadvantages of Batch Culture.

- Culture ageing, and more importantly differentiation, can be a specific problem, especially so with growth-related products;
- Build up of toxic metabolites can restrict cell growth and product formation;
- Initial substrate concentrations may have to be limited due to problems with inhibition and repression effects, therefore affecting the amount of product that can be obtained from such simple systems;
- Batch-to-batch variability;
- The use of batch cultures in industrial systems can lead to an increased nonproductive period due to down time required for cleaning, resterilization, filling and cooling of equipment;
- If using the organism from one bioprocess to seed another culture, degeneration or differentiation may occur, which could affect the bioprocess and product formation;
- Cellular autolysis may occur during the decline and stationary phase, affecting the amount of product, its composition and potentially adding to downstream processing challenges due to release of autolytic breakdown products, activation of proteases;
- From a physiological viewpoint the use of batch cultures actually contributes greatly to the complexity of the experiments since the cell population is heterogeneous and constantly changing. This makes the use of such systems for clearly identifying cause and effect relationships in cell physiology rather unattractive.

2.6 Fermentator type: fermenter design.

The bioreactor should be capable of the following as a minimum:

- Aseptic production for extended periods of time;
- Meeting the local containment regulations;
- Monitoring and controlling the following parameters:
    1. pH by either acid/base addition or CO_2/base addition;
    2. mixing such that the culture remains in suspension and DO_2 is maintained; all this should be achieved without damage to the organisms;
    3. temperature regulation;
    4. sterile sampling capability.

Irrespective of their configuration, industrial bioreactors for sterile operations are designed as pressure vessels, capable of being sterilized *in situ* with saturated steam at a minimum gauge pressure of 0.11 MPa. Typically, the bioreactor is designed for a maximum allowable working pressure of 0.28-0.31 MPa (gauge) and a temperature of 150-180°C. The vessels are designed to withstand a full vacuum. Modern commercial fermenters are predominantly made of stainless steel. Type 316L stainless steel is preferred, but the less expensive Type 304L (or 304) may be used in less corrosive situations. Fermenters are typically designed with clean-in-place capability.

A typical submerged-culture vessel has the features shown in figure 2.6. Sight glasses in the side and top of the vessel allow for easy viewing. The top sight glass can be cleaned during fermentation, using a short-duration spray of sterile water derived from condensed steam. An external lamp is provided, to light the vessel through the sight glass or a separate window. The vessel has ports for sensors of pH, temperature and dissolved O_2. A steam-sterilizable sampling valve is provided. Connections for the introduction of acid and alkali (for pH control), antifoam agents, substrate and inoculum are located above the liquid level in the bioreactor vessel. Additional ports on the top support a foam-sensing electrode, a pressure sensor and sometimes other instruments. Filter-sterilized gas for aeration is supplied through a submerged sparger. Sometimes CO_2 or ammonia may be added to the aeration gas, for pH control.

A harvest valve is located at the lowest point on the fermenter. A mechanical agitator, entering from either the top or the bottom, may be used. The agitator shaft supports one or more impellers, of various designs (Fig. 2.7). A high-speed mechanical foam breaker may be provided at the top of the vessel, and waste gas may exit through the foam

breaker. Commonly, the exhaust gas line also has a heat exchanger, to condense and return water in the gas to the fermenter. The top of the fermenter is either removable or provided with a manhole. A port on the top supports a 'rupture disc' that is piped to a drain. The disc is intended to protect the vessel in the event of a pressure build-up. The fermentation vessel is jacketed for heat exchange, and the jacket may be covered with fibreglass insulation and a protective metal shroud. Additional surfaces for heat exchange, typically coils, may be located inside the vessel.

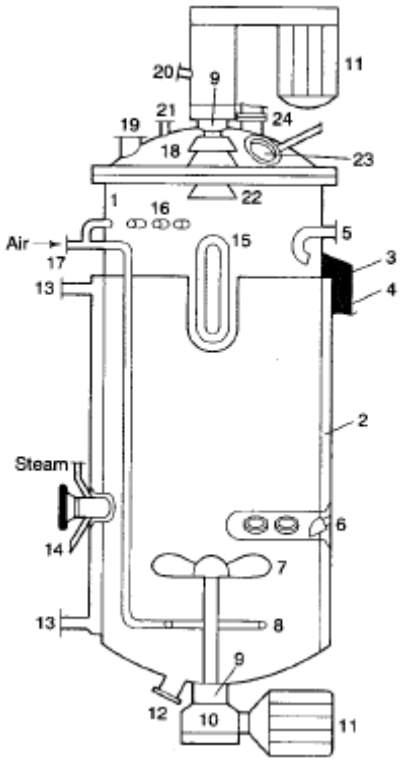


Fig. 2.6: A typical submerged-culture fermenter. (1) Reactor vessel. (2) Jacket. (3) Insulation. (4) Protective shroud. (5) Inoculum connection. (6) Ports for sensors of pH, temperature and dissolved O_2. (7) Agitator. (8)Gas sparger. (9) Mechanical seal. (10) Reducing gearbox. (11) Motor. (12) Harvest nozzle. (13) Jacket connections. (14) Sample valve with steam connection. (15) Sight glass. (16) Connections for acids, alkalis and antifoam agents. (17) Air inlet. (18) Removable top. (19) Medium feed nozzle. (20) Air exhaust nozzle (connects to condenser, not shown). (21) Instrumentation ports for foam sensor, pressure gauge and other devices. (22) Centrifugal foam breaker. (23) Sight glass with light (not shown) and steam connection. (24) Rupture disc nozzle. Vertical baffles are not shown. Baffles are mounted on brackets attached to the wall. A small clearance remains between the wall and the closest vertical edge of the baffle (Y. Chisti, 1999).

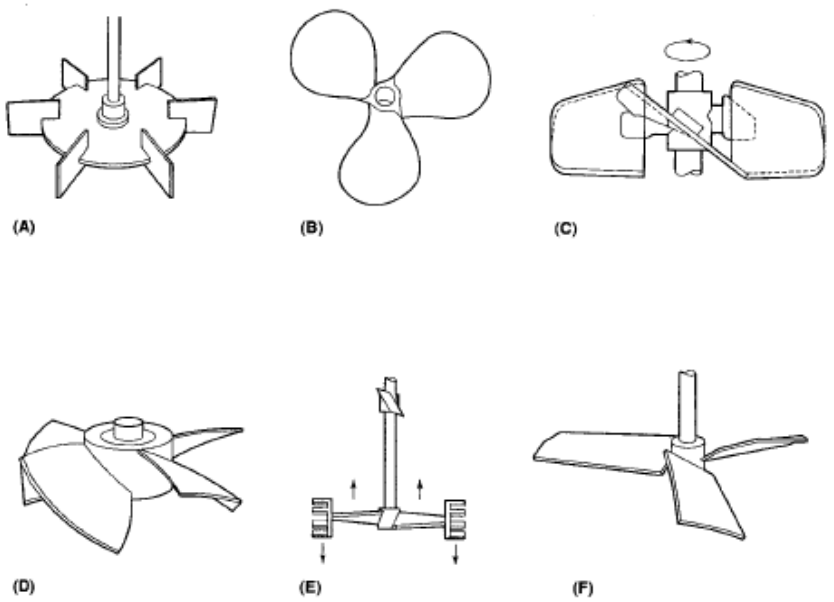


Fig. 2.7: Impellers for stirred-tank fermentars. (A) Rushton disc turbine (radial flow). (B) Marine propeller (axial flow). (C) Lightnin'hydrofoil (axial flow). (D) Prochem hydrofoil (axial flow). (E) Intermig (axial flow). (F) Chemineer hydrofoil (axial flow) (Y. Chisti, 1999).

Considerations in selecting industrial fermentes are:

1. Nature of substrate solid, liquid, suspended slurry.
2. Flow behaviour (rheology), broth viscosity and type of fluid (e.g. Newtonian, viscoelastic, pseudoplastic, Bingham plastic).
3. Nature and amount of suspended solids in broth.
4. Whether fermentation is aerobic or anaerobic, and O_2 demand.
5. Mixing requirement.
6. Heat-transfer needs.
7. Shear tolerance of microorganism, substrate and product.
8. Sterility requirements.
9. Process kinetics, batch or continuous operation, single-stage or multistage fermentation.
10. Desired process flexibility.
11. Capital and operational costs.
12. Local technological capability and potential for technology transfer.

2.7 Food Drying.

Drying is a process used for preservation and/or weight reduction. Drying of food materials is commonly performed before they are stored for long periods of time, and this storage can occur without need for refrigeration or aseptic packaging. Transportation of dried foods is more efficient than for wet foods because, in addition to the absence of refrigeration, energy need not be spent to transport water, and dried materials usually occupy less space than their wetted counterparts. This apparent energy advantage could be overwhelmed by the energy required to dry the materials in the first place; so one of the major challenges in drying is to perform it as economically as possible.

There are a number of classes of microorganisms that are important in spoilage of food and other agricultural materials. Some of these appear in Tab. 2.1, along with figures related to moisture content that show the minimum relative dryness at which they will grown. Bacteria, moulds, yeasts, and fungi all appear. Not all of these classes of microorganisms grow in all materials, but many of them are common in food spoilage.

Group	**Minimum Equilibrium Water Vapour Pressure**
Normal Bacteria	0.91
Normal yeasts	0.88
Normal moulds	0.80
Halophilic bacteria	0.75
Xerophilic fungi	0.65
Osmophilic yeasts	0.60

Tab.2.1: Microorganisms groups important in spoilage and their minimum moisture conditions (A.T. Johnson, 1999).

Drying of bioproducts is a mass transfer process resulting in the removal of water moisture or moisture from another solvent, by evaporation from solid, semi-solid or liquid (hereafter product) to end in a solid state. To achieve this, there must be source of heat, and a sink of the vapour thus produced. In bioproducts (food, grains, vaccines), and pharmaceuticals, the solvent to be removed is almost invariably water.

In the most common case, a gas stream, e.g., air, applies the heat by convention and carries away the vapour as humidity. Other possibilities are vacuum drying, where heat supplied by contact conduction or radiation (or microwaves) while the produced vapour is removed by the vacuum system. Another indirect technique is drum drying, where a heated surface is used to provide the energy and aspirators draw the vapour outside the drum.

Freeze drying or lyophilisation is a drying method where the solvent is frozen prior to drying and is then sublimed, i.e., passed to the gas phase directly from the solid phase, below the melting point of the solvent. Freeze drying is often carried out under high vacuum to allow drying to proceed at a reasonable rate. This process avoids collapse of the solid structure, leading to a low density, highly porous product, able to regain the solvent quickly. In biological materials of foods, freeze drying is regarded as one of the best if not the best method to retain the initial properties. It was first used industrially to produce dehydrated vaccines, and to bring dehydrated blood to assist war casualties. Now freeze drying is increasingly used to preserve some foods, especially for backpackers going to remote areas. The method may keep protein quality intact, the same as the activity of vitamins and bioactive compounds.

In turn, the mechanical extraction of the solvent, e.g., water by centrifugation, is not considered “drying”. The ubiquitous term dehydration may mean drying of water-containing products as foods, but its meaning is more vague, as it is also applied for water removal by osmotic drive from a salt or sugar solution. In medicine, dehydration is the situation by which a person loses water by respiration, sweating and evaporation and does not incorporate, for whatever reason, the “make-up” water required to keep the normal physiological behaviour of the body.

Many product of the biochemical industry such as vaccines, enzymes, pharmaceuticals, etc., have to be dehydrated for preservation. Dry products keep well and are easy to

package and transport. Spray drying technology are particularly important for thermolabile, biologically active products.

2.7.1 Method of drying.

In a typical phase diagram (Fig. 2.8), the boundary between gas and liquid runs from the triple point to the critical point. Regular drying is the green arrow, while supercritical drying is the red arrow and freeze drying is the blue.

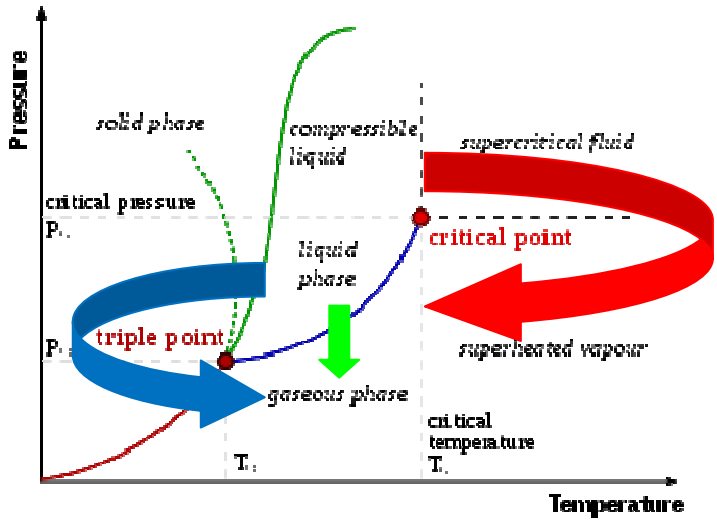


Fig. 2.8: Typical phase diagram. The boundary between gas and liquid runs from the triple point to the critical point. Regular drying is the green arrow, while supercritical drying is the red arrow and freeze drying is the blue.

- Application of heated air (convective or direct drying). Air heating reduces air relative humidity, which is the driving force for drying. Besides, higher temperatures speed up diffusion of water inside the solids, so drying is faster. However, product quality considerations limit the applicable rise to air temperature. Too hot air almost completely dehydrates the solid surface, so internal pores shrink and almost close, leading to crust formation or "case hardening".

- Indirect or contact drying (heating through a hot wall), as drum drying, vacuum drying.
- Dielectric drying (radiofrequency or microwaves being absorbed inside the material) It is the focus of intense research nowadays. It may be used to assist air drying or vacuum drying. Researchers have found that microwave finish drying speeds up the otherwise very low drying rate at the end of convective drying.
- Freeze drying is increasingly applied to dry foods, beyond its already classical pharmaceutical or medical applications. It keeps biological properties of proteins, and retains vitamins and bioactive compounds. Pressure may be reduced by a vacuum pump. If using a vacuum pump, the vapor produced by sublimation is removed from the system by converting it into ice in a condenser, operating at very low temperatures, outside the freeze drying chamber.
- Supercritical drying (superheated steam drying) involves steam drying of products containing water. Strange as it seems, this is possible because the water in the product is boiled off, and joined with the drying medium, increasing its flow. It is usually employed in closed circuit and allows a proportion of latent heat to be recovered by recompression, a feature which is not possible with conventional air drying, for instance. May have potential for foods if carried out at reduced pressure, to lower the boiling point.
- Natural air drying takes place when materials are dried with unheated forced air, taking advantage of its natural drying potential. The process is slow and weather-dependent, so a wise strategy "fan off-fan on" must be devised considering the following conditions: Air temperature, relative humidity and moisture content and temperature of the material being dried. Grains are increasingly dried with this technique, and the total time (including fan off and on periods) may last from one week to various months, if a winter rest can be tolerated in cold areas.

2.7.2 Applications of drying.

To perform the drying of fermented substrate up to 20% of solid is suitable major efficient to use spray dryer.

2.7.2.1 Spray drying.

Spray drying is an important technique to produce dried powders. The principle is that a pumpable feed is first atomized, i.e, converted in a fog of droplets of about 100 micrometers in diameter, which dry very fast while falling by gravity, accompanied by heated air. Spray drying is a method for rapid, continuous, drying of solutions, emulsions and slurries. Pressure or centrifugal atomizers or gas-liquid jets are used to generate a fine spray of solution droplet which are brought into continuous contact with hot air in a large chamber (fig. 2.9). Large droplet surface area and small droplet size ensure high evaporation rates so that drying times are but a few seconds. The flow of air is usually cyclonic. The dimensions of the drier must be such that the droplets do not reach the walls until sufficiently dry to prevent sticking and burn-on. A drying chamber tends to be quite large: 1-10 m in diameter being common. The dry powder settles to the bottom from where it is removed either pneumatically or mechanically, or by a combination of these methods.

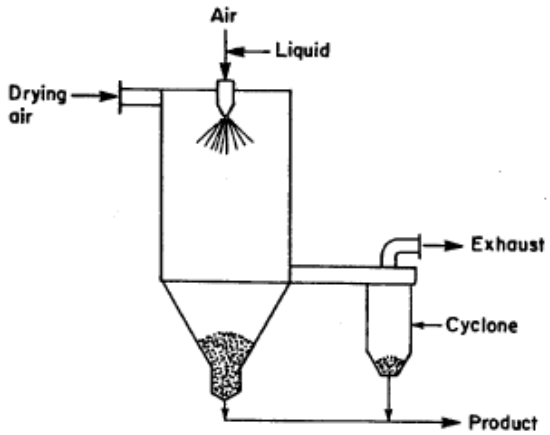


Fig. 2.9: Schematic of a spray drying (Y. Chisti and Murray Moo-Young, 1999).

Advantages of spray driers are: continuous operation, powder product requiring no further size reduction and rapid drying which leads to good product quality particularly for heat-labile materials but relatively low thermal efficiency is a limitation. Aseptic spray drying equipment is available. All the air used is filter sterilized and the drying and solids-handling chambers operate under slight positive pressure. The installations can be operated leak-tight and are sterilizable. Antibiotics such as streptomycine sulphate for direct injection can be spray dried. Highly heat-labile products like some enzymes and blood sera can be successfully spray drier. Microorganisms may be spray-drier for preservation and use as SCP.

Milk powder is possibly the most popular product, followed by instant coffee. Tomato powder is becoming very important. On the other hand, washing powder is an example product of the chemical process industry. The production of dehydrated natural flavors and essences is very important and is growing together with encapsulation, a technique devised to trap a volatile, but large molecule (as the flavor compound) inside a dry particle, the walls of which develop on drying and are more permeable to the water flux than to the flux of the larger volatiles. This principle of selective diffusion was first developed by the Dutch researcher Thijssen, in Eindhoven, during the 1970's, and is improved by adding maltodextrins (a product from controlled starch hydrolysis) to the dryer feed. Spray dryers differ in the type of atomizer, the relative directions of air and product flows, the chamber design, type of drying agent (air, nitrogen) and system characteristics (closed or open circuit), among other features. Equipment can be very large of up to 20 m tall.

2.8 Bioprocess control.

In order to ensure optimal functioning of a bioprocessing plant several processing parameters need to be monitored and controlled. Temperature, pH, product and substrate concentrations, dissolved oxygen, and material flows are a few of those which may have to be followed over time and manipulated in some predetermined way so as to

obtain the desired product yields at minimal cost. Computer-based control systems are increasingly encountered in biochemical processing plants and operations such as in-place cleaning, filling, and sterilization sequences are often fully automated. Control of the biochemical reactor or fermenter in generally limited to control of pH, temperature, agitation rate, foam and dissolved oxygen. Several options are often available to control the various parameters. Selection of specific methods depends on the process. In microbial fermentations, agitation speed and air flow rate are used in combination. In animal cell culture processes, a common approach is to vary the composition of the aeration gas while keeping the total flow rate constant. Control of foam is done either by adding chemical antifoam breakers (fig. 2.10). A foam sensor provides the signal for control.

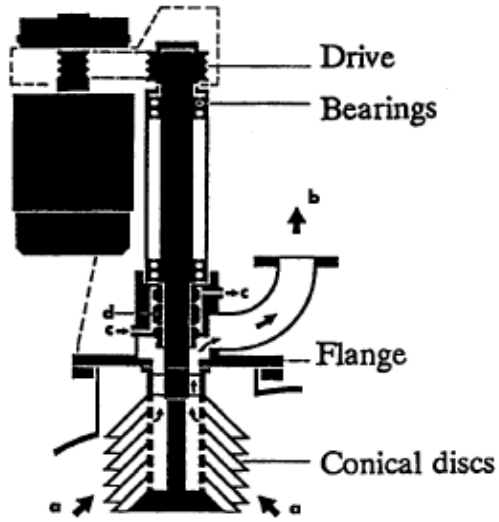


Fig. 2.10: A mechanical foam breaker. The foam enters the rotating conical discs at (a) and is separated into gas and liquid by the centrifugal force. The liquid spins into the bioreactor and liquid-free gas exhausts through the nozzle (b). The mechanical seal (d) is lubrificated by sterile cooling water (c) (Y. Chisti and Murray Moo-Young, 1999).

A typically instrumented fermenter is show in figure 2.11. More extensive control of fermentation processes is desiderable but it is restricted by two main factors: (i) the availability of online sensors to measure the biological and physicochemical parameters needed to follow the progress of fermentation remains limited; and (ii) our limited

ability to interpret the available information in the context of the biological system so that the information obtained can be used as a basis for control.

Sophisticated control of fermentation systems presupposes the existence of mathematical description - or model- of the process. The kinetics of growth and product formation in combination with physical system characteristics form a set of equations which constitute the process model. One of the aims of control is optimisation of the process. In batch or fed-batch cultures, profiles of progressive changes in environmental conditions (temperature, pH, dissolved oxygen, substrate concentration) are determined for maximization of product yield. In continuous cultivation, optimisation is used to select an environmental regime for maximum biomass or product formation.

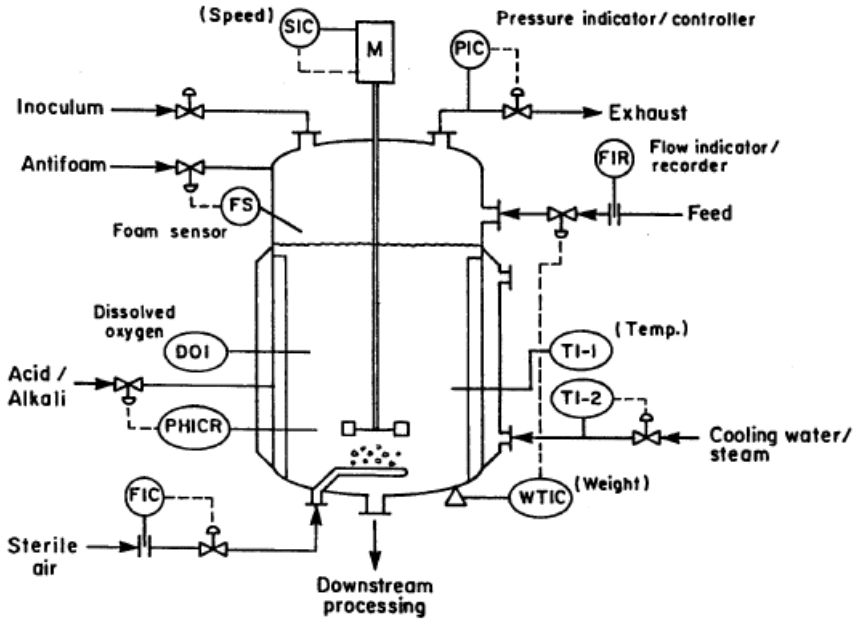


Fig.2.11: Typical fermenter instrumentation (Y. Chisti and Murray Moo-Young, 1999).

Chapter III

Selection of bacterial strain

Different type of bacterial strain are studied in order to select the best microorganisms performance.
In particular the following lactobacilli have been tested:

1. *L. paracasei* 6/5
2. *L. paracasei 10/2 (=CBA-L74)*
3. *L paracasei 6/11*
4. *L. casei 4/3*
5. *L. paracasei 5/1*

Different analyses have been performed to test their capability to ferment a water suspension containing rice flour and wheat flour at different concentration (Tab. 3.1).

3.1 Experimental apparatus and analytical methods

Fermentation tests have been carried out in a 5L batch reactors containing water at constant temperature of 37°C.
The main features of fermentator are:

1 Ready-to-use packages for microbial or cell cultures applications;
2 Inclusive Notebook PC for operation;
3 Measure and control of temperature, pH, PO_2, stirrer speed, gas mixing, foam

level and substrate concentration;

4 4-gas mixing system with individual gas flow path for cell culture packages;

5 Powerful PC operating software - capable of handling up to four units (Fig. 3.1).

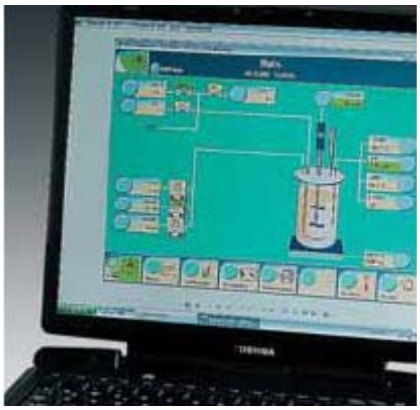


Fig. 3.1: PC operating software.

Laboratory plant consist of a 5L fermentation reactor (Biostat A Plus) (Fig. 3.2) equipped with an acquisition unit suitable interfaced to a PC to perform the elaboration of experimental signals (Fig. 3.3).

Fig. 3.2: Vessel with 5L working volume.

Fig. 3.3: Laboratory plan (Biostat A Plus).

The Biostat A Plus is a compact autoclavable fermentor/bioreactor system specially designed for lab scale R&D applications.

Fermentator is equipped with following sensors:

- pH, by pH meter Expandable Ion Analyzer EA920 – Orion Research (Appendix A.1) in range 2 ÷ 7;
- PO_2, by O_2-meter in range 0 ÷ 200 %;
- Rotational speed 20 ÷ 200 rpm;
- Temperature 10 ÷ 100°C.

Details description of the pH and of measurement systems is shown in Appendix A.

Firstly fermentation runs are analysed only by pH. For each run optimal value of pH, determined by lowest values, pointed out suitable concentration of the flour in the water suspensions.

3.2 Experimental results.

Preliminary tests were performed in order to pointed out optimum concentration of flour (rice and wheat) in water suspension.

This concentration is determined measuring pH level and microbial growth during fermentation tests. Low values of pH together with high values of CFU counts can be used as indication of a good performance of the fermentation.

Values of pH and CFU counts must be linked to low values of O_2 partial pressure in fermented suspension confirming the growth of only anaerobic microorganisms i.e. Lactobacilli.

pH values and partial pressure of O_2 is automatically measured and acquired by system at 0, 4, 6, 18 and 20 hours after the start of fermentation. In the case of some strains the acquisition is performed only at the start and at the end of fermentation that occur at about 20 hours.

In the following table 3.1 the values of pH measured for the strains tested are shown.

STRAIN	MIX	RICE SUSPENSION (pH)					WHEAT SUSPENSION (pH)				
		T_0	4 h	6 h	18 h	20 h	T_0	4 h	6 h	18 h	20 h
6/5	0%	6,36	5,81	5,42	3,81	3,59	5,92	-	-	3,84	3,71
	10%	6,22	5,97	5,53	3,80	3,50	5,89	-	-	3,89	3,78
	20%	6,17	5,95	5,52	3,79	3,48	5,89	-	-	3,91	3,79
	40%	6,12	5,97	5,63	3,79	3,45	5,88	-	-	3,89	3,78
	60%	6,15	5,96	5,63	3,77	3,44	5,89	-	-	3,89	3,78
	100%	6,12	5,96	5,74	3,81	3,54	5,87	-	-	3,91	3,79
10/2	0%	6,36	5,83	5,73	3,62	3,49	5,92	-	-	3,79	3,75
	10%	6,22	5,95	5,71	3,90	3,52	5,89	-	-	3,79	3,76
	20%	6,17	5,93	5,73	3,89	3,46	5,89	-	-	3,78	3,76
	40%	6,12	5,97	5,71	3,91	3,51	5,88	-	-	3,79	3,76
	60%	6,15	5,99	5,72	3,88	3,52	5,89	-	-	3,80	3,78
	100%	6,12	6,01	5,73	3,89	3,59	5,87	-	-	3,81	3,79
6/11	0%	6,36	-	-	-	3,57	5,92	-	-	-	3,70
	10%	6,22	-	-	-	3,56	5,89	-	-	-	3,71
	20%	6,17	-	-	-	3,59	5,89	-	-	-	3,71
	40%	6,12	-	-	-	3,61	5,88	-	-	-	3,72
	60%	6,15	-	-	-	3,61	5,89	-	-	-	3,74
	100%	6,12	-	-	-	3,62	5,87	-	-	-	3,74
4/3	0%	6,36	-	-	-	3,73	5,92	-	-	-	3,52
	10%	6,22	-	-	-	3,75	5,89	-	-	-	3,56
	20%	6,17	-	-	-	3,74	5,89	-	-	-	3,56
	40%	6,12	-	-	-	3,71	5,88	-	-	-	3,57
	60%	6,15	-	-	-	3,79	5,89	-	-	-	3,56
	100%	6,12	-	-	-	3,76	5,87	-	-	-	3,58
5/1	0%	6,36	-	-	-	3,48	5,92	-	-	-	3,49
	10%	6,22	-	-	-	3,48	5,89	-	-	-	3,48
	20%	6,17	-	-	-	3,48	5,89	-	-	-	3,49
	40%	6,12	-	-	-	3,49	5,88	-	-	-	3,49
	60%	6,15	-	-	-	3,49	5,89	-	-	-	3,49
	100%	6,12	-	-	-	3,48	5,87	-	-	-	3,51

Tab. 3.1: Fermentation tests about pH analyses at different concentration of flours.

By table analyses it is clear that optimal condition (lowest pH value) in rice fermentation are obtain at a concentration in the range 40% ÷ 60% and after 18-20 h of process, while in the wheat fermentation optimum conditions are at 40% for time of process of 18-20h.

Coupled the results of table 3.1 can be shown microbial growth for the same fermentation tests.

The microbial growth is derived by CFU count sowing Petri Plates with fermented suspension sampled at the end of fermentation (20 h, lowest pH value).

In the following table 3.2 are shown CFU counts in the case of rice and wheat suspension and compared to initial concentration of microorganisms.

STRAIN	**BACTERIAL**	**UFC inoculum**	**UFC rice**	**pH rice**	**UFC wheat**	**pH wheat**
6/5	L. paracasei	$2{,}50*10^{7}$	$1{,}80*10^{9}$	3,59	$6{,}60*10^{7}$	3,84
10/2	L. paracasei	$3{,}00*10^{6}$	$2{,}40*10^{9}$	3,49	$5{,}50*10^{8}$	3,79
6/11	L. paracasei	$4{,}40*10^{7}$	$5{,}00*10^{7}$	3,57	$2{,}00*10^{8}$	3,70
4/3	L. casei	$4{,}30*10^{7}$	$9{,}00*10^{8}$	3,73	$4{,}40*10^{8}$	3,52
5/1	L. paracasei	$3{,}40*10^{7}$	$1{,}30*10^{9}$	3,48	$9{,}00*10^{8}$	3,49

Tab. 3.2: UFCs and pH analyses at optimal fermentation conditions.

CFU data are compared in Fig 3.4 showing overall better performed of Lactobacillus Paracasei 10/2.

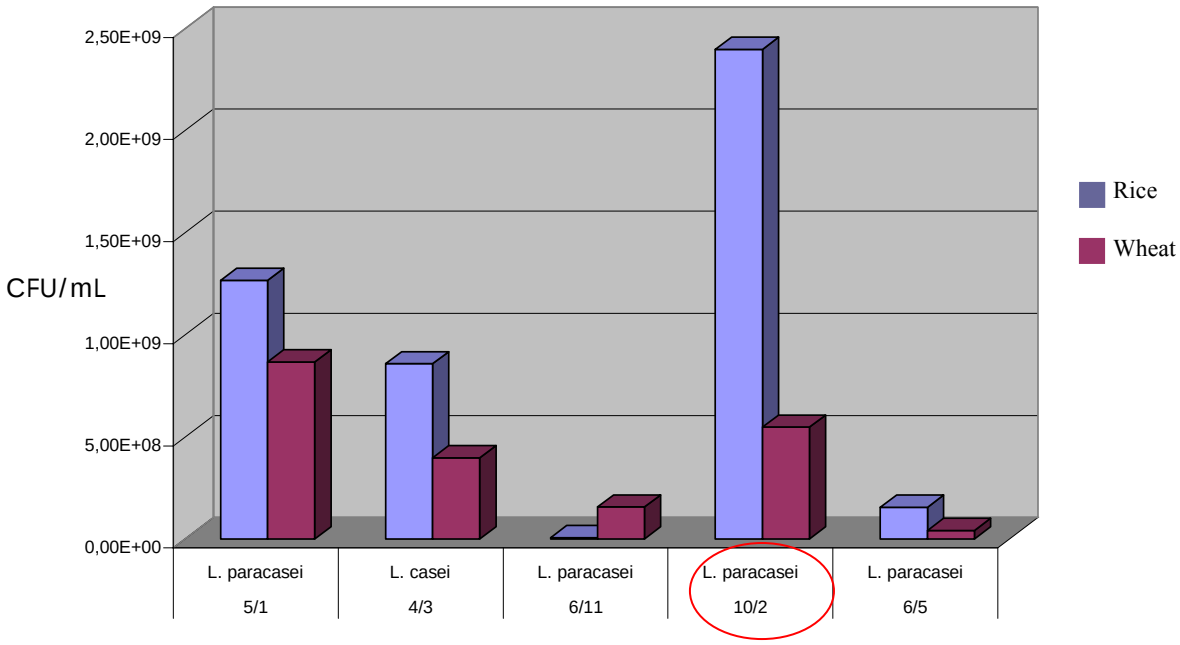


Fig. 3.4: Compare to CFU for different bacterial strains.

Moreover the fermentation performed Lactobacillus strain 10/2 shows a good organoleptic properties of the fermented product.

Chapter IV

Analysis of fermentation of substrate on a lab-scale

In this experimental lab-scale phase the selected strain is used to determine the fermentation protocol.

Fermentation tests run on two different substrates: rice flour/water suspension and commercial skim milk.

Also to skim milk is utilized, as preliminary tests, the same Lactobacillus Paracasei 10/2 but, in this case, any study are performed to select the best microorganisms performance.

4.1 Experimental apparatus and analytical methods.

Lab-scale fermentation experiments carried out in a 5L batch reactor describe in previous chapter III.

To verify the correct proceed to fermentation, different parameters are analyzes:

a) *pH:* at different time of reaction it has taken a sample to analyze reaction advancement between pH value (Appendix A.1);
b) *Bacterial growth:* samples of fermented product have been sowed on Petri Plate in order to measure the growth of lactobacilli.
   MRS agar broth has been used. Experimental methods are indicated in Appendix A.2.
c) *PO_2:* during fermentation process saturation O_2 percentage are measured by O_2-

meter (Appendix A.3);

4.1.1 Rice flour/water suspension.

Experimental tests carried out on rice flour/water suspension in the same optimum concentration that are determined in previous chapter III and with Lactobacillus Paracasei 10/2 that present better performance on rice flour/water suspension as it described in chapter III.

4.1.1.1 Rice flour/water suspension protocol.

Experimental protocol for rice flour/water suspension is characterized by different points:

1 Sterilization of lab-fermenter filled with 1L of stilled water using autoclave at 120°C for 20 min;
2 Sterilization of 500g of rice flour by dry heat at 120°C for 1h in 1L bottle (fig.4.1);
3 Supply of rice sterilized flour in fermenter using sterile funnel and Bunsen burner under moderate agitation of 150 rpm velocity of stirrer;
4 Supply of inoculum in fermenter using Bunsen burner under moderate agitation of 150 rpm velocity of stirrer;
5 Fermentation start;
6 Fermentation temperature = 37°C;
7 Optimum stirring velocity = 150 rpm to avoid sedimentation.

Fig.4.1: 1L bottle to rice flour sterilization.

4.1.1.2 Experimental results.

In the following figures are reported the behaviours of ph, PO_2 and CFU/mL measured during fermentation runs:

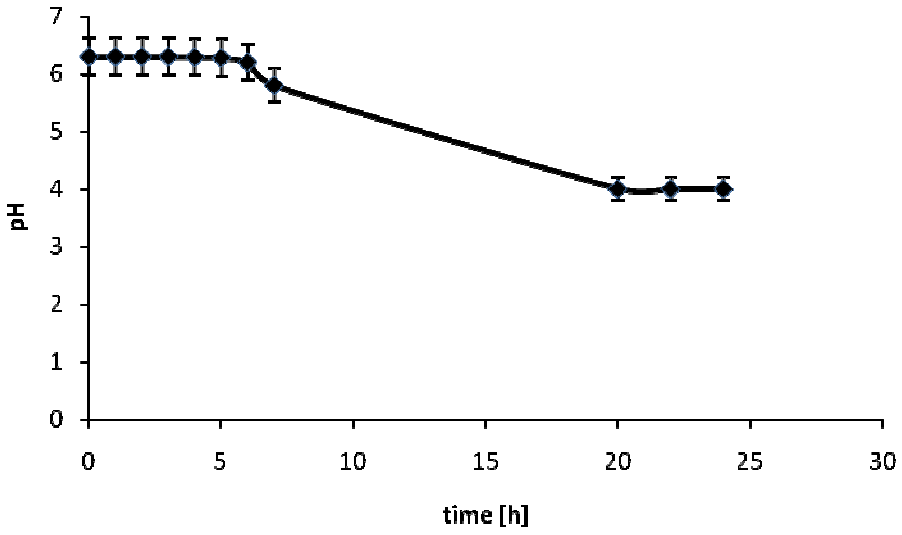


Fig. 4.2: pH profile control during fermentation of rice flour/water suspension (T =37°C, stirrer =150 rpm).

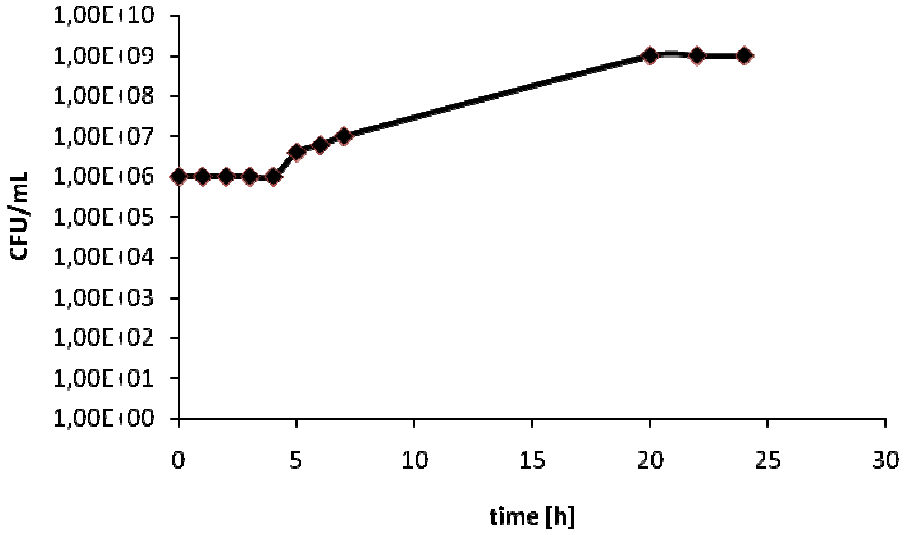


Fig. 4.3: CFU/mL profile during fermentation of rice flour/water suspension (T = 37°C, stirrer = 150 rpm).

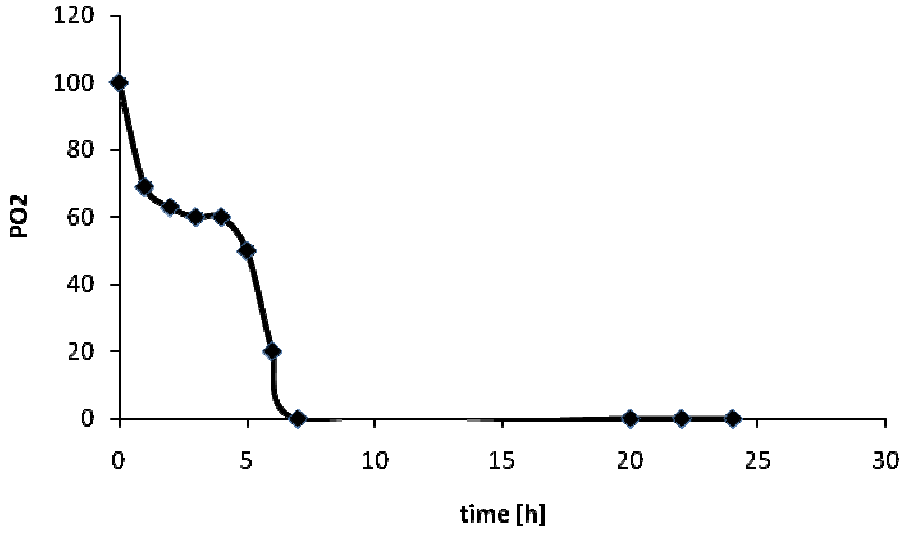


Fig. 4.4: PO_2 profile control during fermentation of rice flour/water suspension (T =37°C, stirrer=150 rpm).

In the previous figures three main growth phases can be recognized; log phase (0 ÷ 5 h), exponential (5 ÷ 20 h) and stationary (20 ÷ 24 h). This behaviours are connected with low values of partial pressure of O_2 measured from 5 to 24 h.

4.1.2 Skim milk.

Experimental tests carried out on commercial skim milk and Lactobacillus Paracasei 10/2 are used.

4.1.2.1 Skim milk protocol.

Experimental protocol for commercial skim milk in 1 L tetrapack is characterized by different points:

1 Sterilization of lab-fermenter using autoclave at 120°C for 20 min;

2 Supply of 1L of milk in fermenter using sterile funnel and Bunsen burner;

3 Milk tyndallisation is obtained directly in the lab fermentator using a specific alternation of heating/cooling steps:
- Heating at 70°C for 30 min;
- Cooling at 30°C for 30 min;
- Heating at 70°C for 30 min;
- Cooling to fermentation temperature (37°C).

4 Supply inoculum in fermenter using Bunsen burner under moderate agitation of 10 rpm;

5 Fermentation start.

6 Optimum stirring velocity = 10 rpm to obtain thermal homogeneity.

4.1.2.2 Experimental results.

In the following figures are reported the behaviours of ph, PO_2 and CFU/mL measured during fermentation runs:

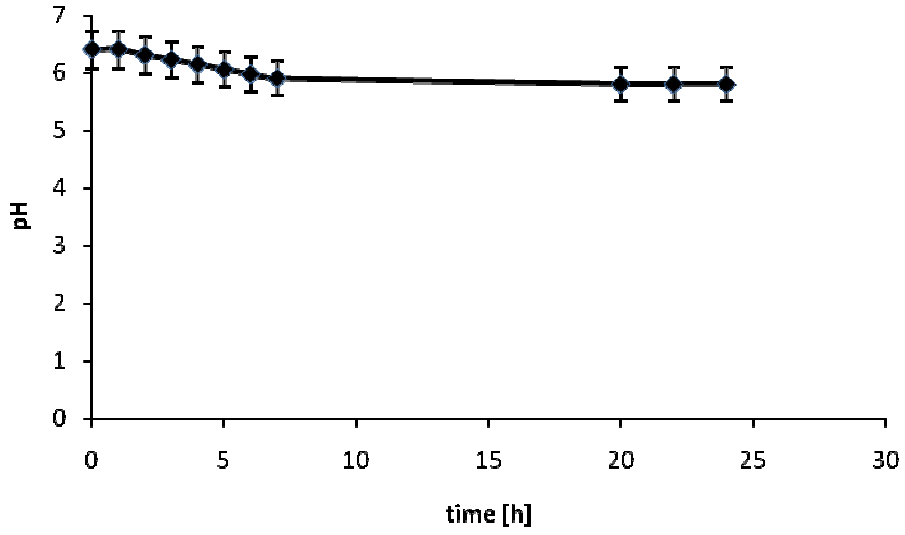


Fig. 4.5: pH profile control during fermentation of skim milk (T = 37°C, stirrer =10 rpm).

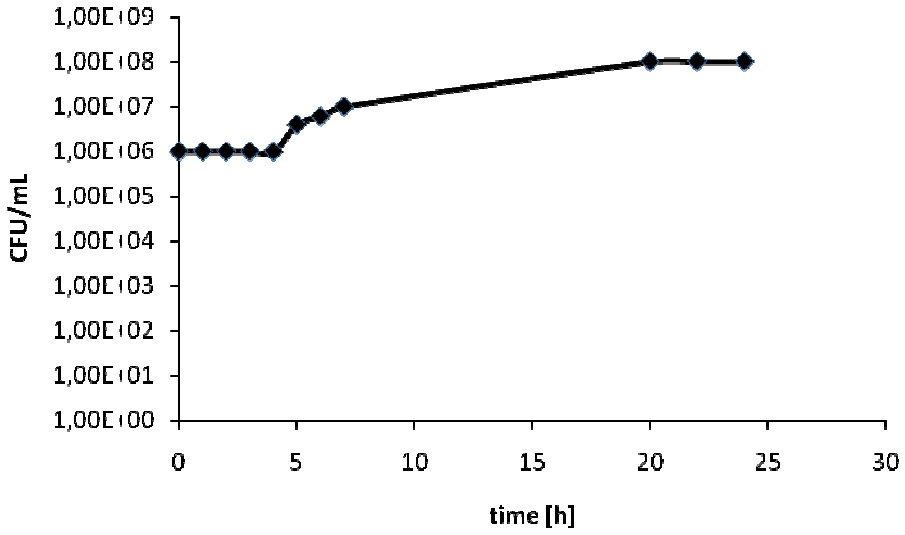


Fig. 4.6: CFU/mL profile during fermentation of skim milk (T = 37°C, stirrer = 10 rpm).

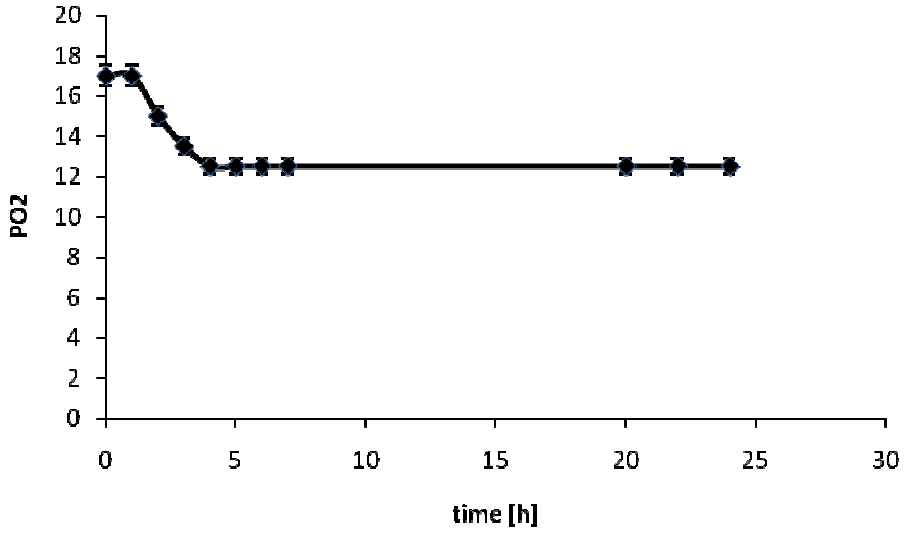


Fig. 4.7: PO_2 profile control during fermentation of skim milk (T = 37°C, stirrer = 10 rpm).

Also in this figures three main growth phases can be recognized; log phase (0 ÷ 4 h), exponential (4 ÷ 20 h) and stationary (20 ÷ 24 h). This behaviours are connected with low values of partial pressure of O_2 measured from 4 to 24 h.

Profile of variables analysed during fermentation can point out the values of pH and CFU count at the end of experimental tests that permit to determining process value of fermentation for both substrates. In tab.4.1 laboratory results are shown.

Substrates	***pH***	***CFU/mL***
Rice flour/water suspension	*4*	*10^8 - 10^9*
Skim milk	*5.8*	*10^8*

Tab 4.1: Laboratory experimental results.

By analyses of laboratory experimental results it is possible confirmed usability of protocols both for rice suspension than for skim milk to scale-up of process.

Chapter V

Development of a fermentation reactor on a pilot-scale

Main activities of this phase have been the development of a fermenter in pilot scale in order to produce a larger amount of fermented product verifying the effectiveness on the protocol developed in lab scale.

The object of scale-up is to reproduce on pilot scale the successful fermentation results achieved in the laboratory. In practice scale-up is quite complex. It is not generally possible to reproduce exactly on the production scale all various parameters for laboratory or pilot scale units. Scale-up is based on the strategy of holding constant only one or two of the several possible parameters at different fermenter scales. The parameter(s) held constant are those which are considered to have the greatest impact on the fermentation; furthermore, the criterion of geometric similarity (i.e. keeping the ratios of corresponding lengths equal on production and pilot-scale units) is not always rigidly adhere to so that small geometric variations may be utilized to advantage as long as they do not result in impracticable behavior.

The scale-up methods which have been most often proposed are as follows:

- Scale-up based on equal power input;
- Scale-up based on equal mixing times;
- Scale-up based on equal oxygen transfer ($k_L a_L$);
- Scale-up based on equal shear rates (or impeller tip speed).

Main activity performed in this sense are:

1. Design of a fermentator in a pilot scale (Cap. V);

2. Development of the fermenter as verifying of mixing process and shear rate (Cap. V);
3. Determination of experimental protocols to be used in pilot scale for rice flour/water suspension and for milk (Cap VI).

5.1 Design and development of pilot scale fermenter.

The design of the pilot plant had the objective of obtaining a fermentation volume of at least 30L in order to obtain an amount of wet fermented product to process with a dehydration treatment to obtain a dry functional product.

Main units in the fermentation apparatus are:

- Vessel: a reaction volume in which can be performed the fermentation. This volume must be interfaced to a mixing system in order to avoid sedimentation of solid mixed in water;
- Mixing system: the impellers are linked to a stirring motor able to 10 rpm up to 250 rpm;
- Thermal system: supplying a hot process fluid able to control the thermal regimes of the fermentation process.

Figure 5.1 shows a scheme of typical fermenter plant, while figures 5.2 shows a photograph of pilot plant in which the single unit can be analyzed. Detail of the parts implemented in each operating unit can are shown in the following figures.

These principal components are connect between additional components those are necessary to the operation of the plant.

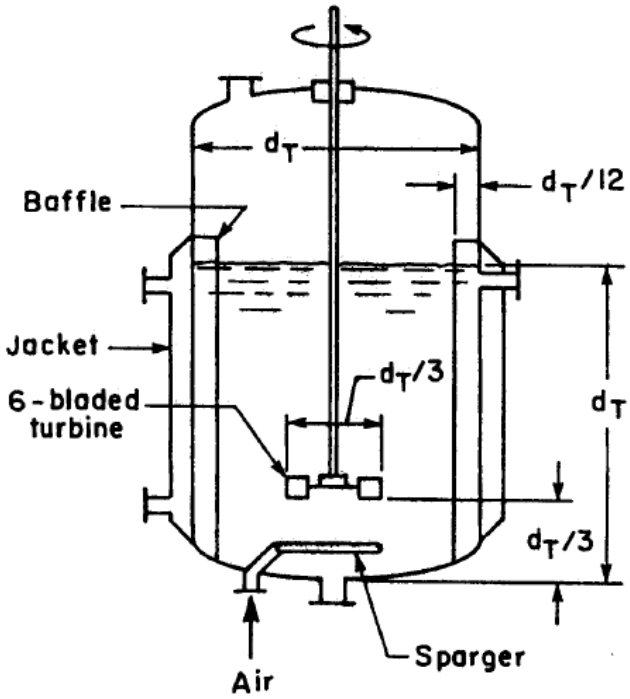


Fig.: 5.1: Scheme of typical fermenter plant.

Fig. 5.2: Pilot plan: Fermenter.

5.1.1 Vessel

The fermentation vessel has a working volume of 30L and is made on AISI 316. On the top an AISI cover (fig.5.3(a)) can be fixed using fast lock in order to seal the reaction volume. On this cover are located several vent ports than can be used to mount vent valves, safety valves or measuring instruments as manometer, thermocouple, pHmeter, DO meters, etc..

On the bottom of the vessel a two position valve is located to sample the fermented product or to evacuate the same (fig.5.3(b), 5.3(c)).

The vessel is of jacketed type in order to supply a suitable amount of a hot/cold process fluid (water) to control the process condition.

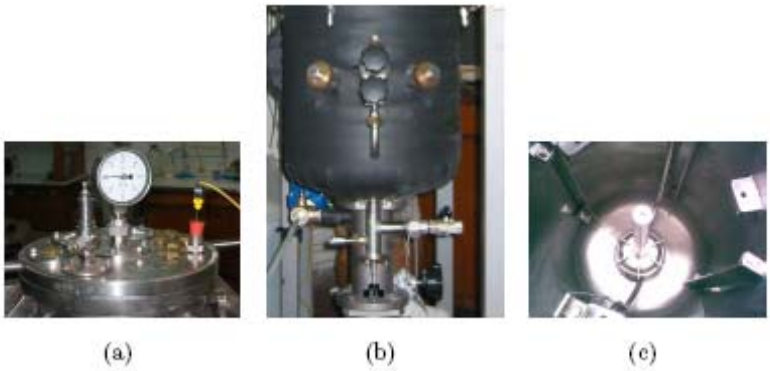


Fig. 5.3: Vessel. (a) Stilled steel cover. (b) Valve to drawing of samples. (c) Impeller.

5.1.2 Mixing system.

The mixing system is composed by a shaft on which are mounted two impellers. First one (fig.5.4(b)) in a 6 plane bladed and is generally positioned at 10 cm from liquid surface and giving preferentially a radial motion to suspension; the second one (fig.5.4(c)) is a two bladed impeller locate at the bottom of the vessel and preferentially gives an axial motion to suspension assuring an optimal agitation to the product avoiding the solid sedimentation.

The rotation of the shaft is assured by a 220 V motor (fig.5.4(a)) able to perform velocity up to 250 rpm.

Velocity of rotation is measured by means a tachymeter (fig. 5.5(a)) and controlled by a mechanical friction wheel mounted on the motor (fig. 5.5(b) and 5.5(c)).

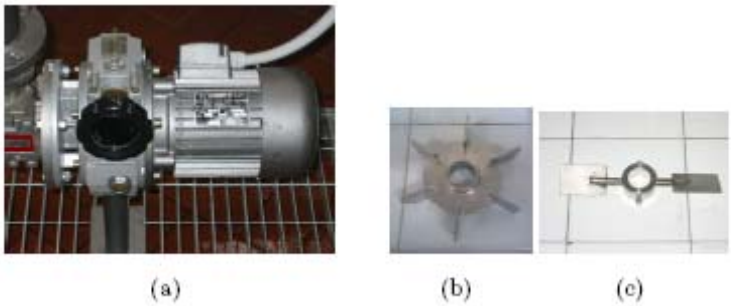


Fig. 5.4: Mixing system. (a)Electric motor. (b) Impeller to radial mixing. (c) Impeller to axial mixing.

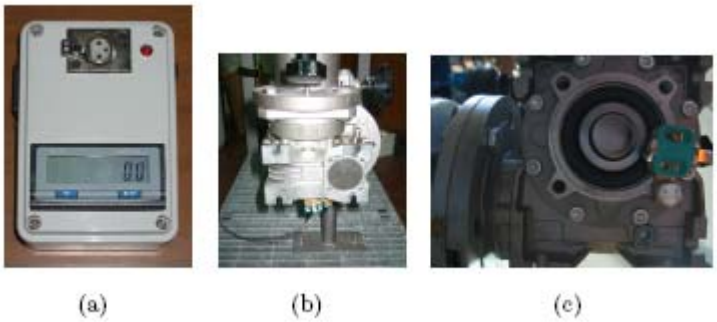


Fig. 5.5: Equipment to measure mixing velocity. (a) Lector to mixing velocity. (b) and (c) Position to sampler.

5.1.3 Thermal system.

Vessel reactor is thermally controlled by thermal system (fig.5.6) able to control the temperature of service fluid (water) that is supplied to the reactor jacket in order to control the substrate temperature at the fermentation value.

Temperatures of the process fluid and of the product are monitored and controlled by a thermocouple (K type) (fig.5.7(b)) interfaced to an on-line acquisition data system (fig.5.7(a)). Temperature data are fedback to control system (fig.5.8).

The temperature of the process fluid supplied to external jacket is controlled by a thermal resistance and by cold water suitably mixed. (fig.5.6(a)).

Fig. 5.6: Thermal system. (a) Tank. (b) Valve to service fluid. (c) Position to sampler.

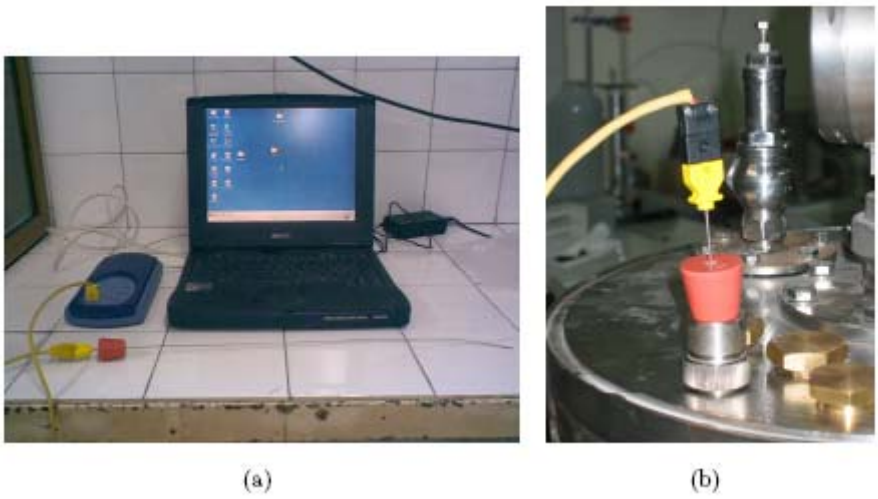


Fig. 5.7: Acquisition data system. (a) Software. (b) Sampler located in vessel.

Fig. 5.8: Controller system.

5.2 Development of fermenter.

Before to perform the fermentation runs, in order to verify that vessel operates in a CSTR regime, it is necessary to control that the concentration of the solid and the temperature in the suspension, during stirred fermentation performed using, are the same in all point of the reactor.
In the following paragraph, the fluid-dynamic characterization of the fermentation vessel has been shown.

5.2.1 Fluid-dynamic characteristics of fermentation volume.

Pilot plan are studied about its mixing system, in particular ideal mixing parameters are object of mixing experiments.
The study are about a type of mixing system and rotational speed.
Fermentation substrate consist in rice flour/water suspension and in skim milk, for this reason in particular for suspension it is necessary to verify that solid concentration is uniform in all product such as temperature. Therefore mixing speed must be optimum to permit vertical movement that inhibit sedimentation, but it must be low to minimize the oxygen passage by gas to suspension (the fermentation is anaerobic).

By analyses of concentration and temperature in more positions of product at different rotating speed it is possible define optimum mixing parameters and optimum position of two different impellers.

For both substrates, in order to have indications on solid concentration and on suspension temperature, a sampling of the products and a measure of the temperature has been performed, following the schemes shown in figure 5.9.

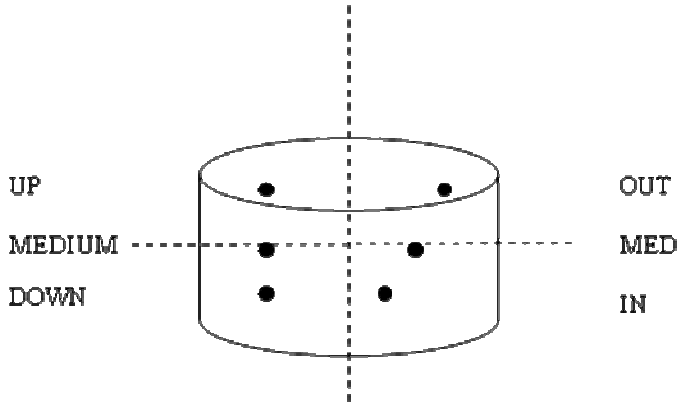


Fig. 5.9: Diagram describe the position of sampler in the vessel.

Plotting this variables vs stirring velocity is possible to point out the velocity at which a completely mixing has been reached.

Measures of solid concentration and of temperature performed in the case of two different products are shown in the following figures 5.10 (solid concentration only for rice/water suspensions), 5.11 ÷ 5.14 and evidenced that CSTR regimes are obtained for stirring velocities above 90 rpm respectively both for the case of the rice/water suspension than milk.

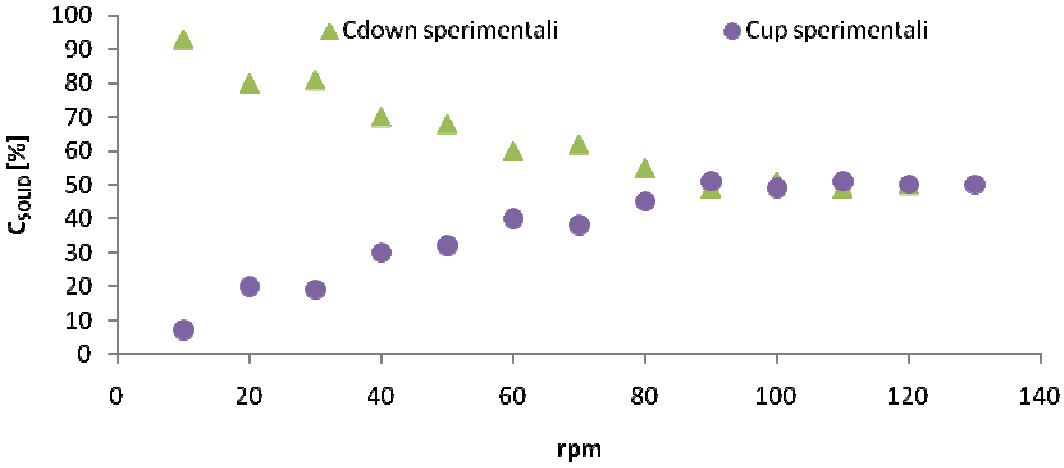


Fig. 5.10: Diagram represent variation of concentration to rpm in rice flour/water suspension.

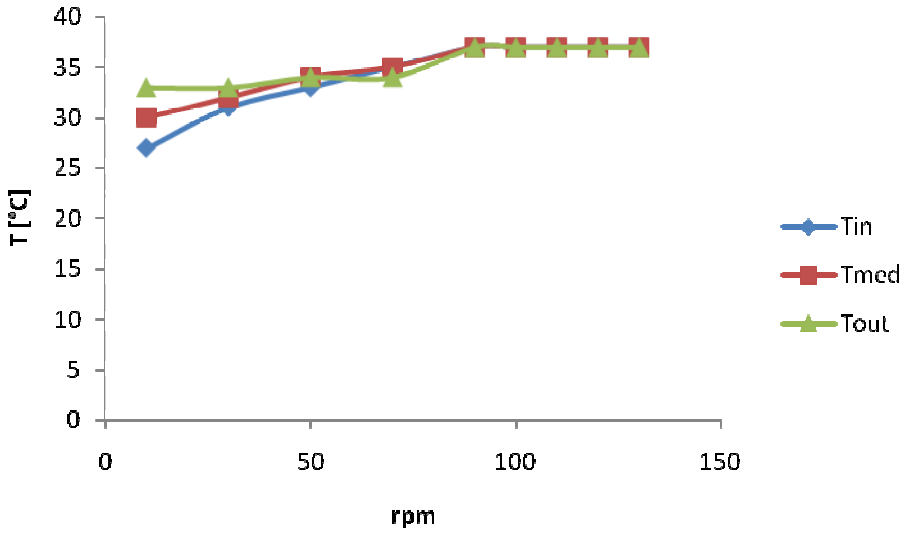


Fig. 5.11: Diagram represent variation of temperature to rpm, in radial direction, for rice flour/water suspension.

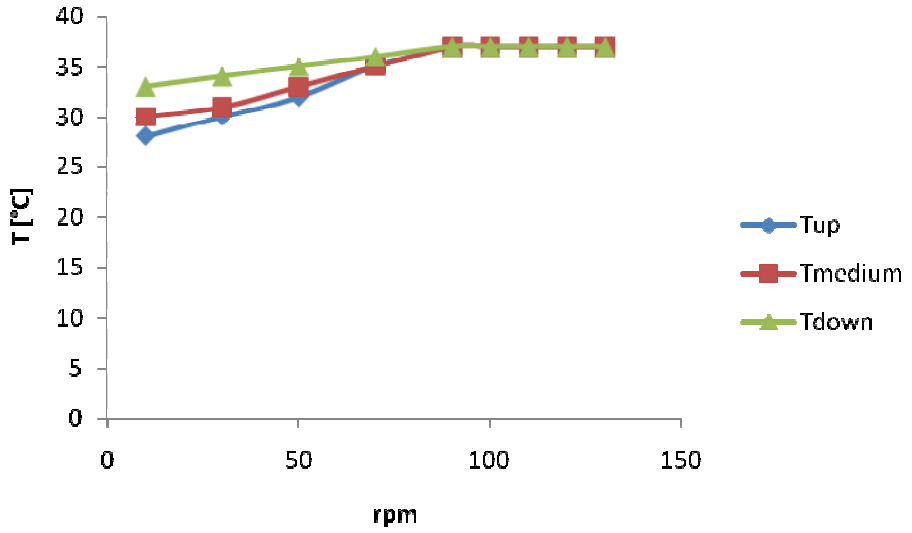


Fig. 5.12: Diagram represent variation of temperature to rpm, in axial direction, for rice flour/water suspension.

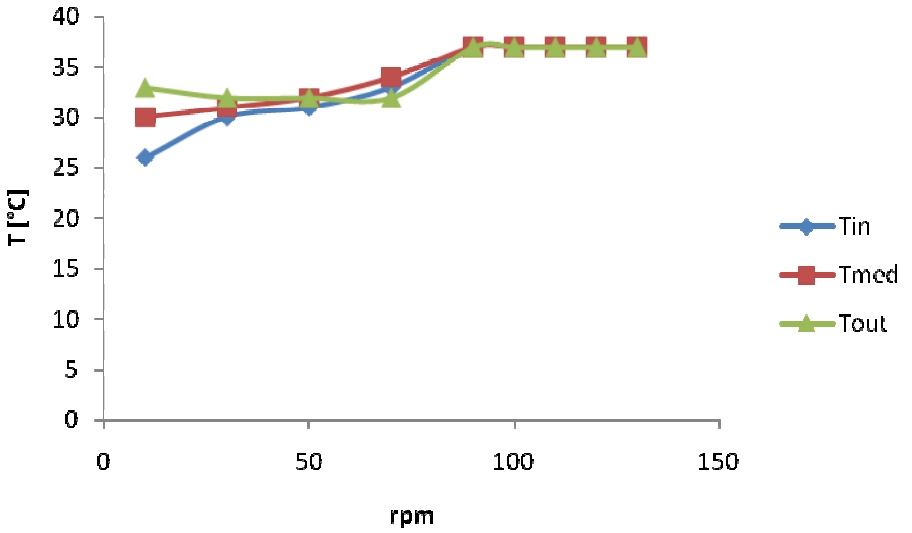


Fig. 5.13: Diagram represent variation of temperature to rpm, in radial direction, for skim milk.

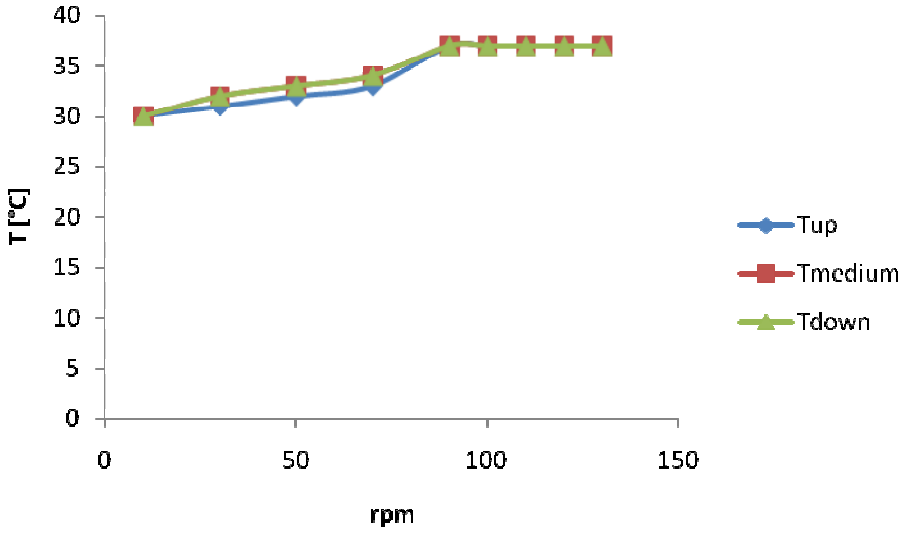


Fig 5.14: Diagram represent variation of temperature to rpm, in axial direction, for skim milk.

Chapter VI

Analysis of fermentation of substrate on a pilot-scale

Basic protocols pointed out in lab-scale fermentation have been used also for preliminary pilot fermentation, taking into an account the minimum stirring velocity point out in fluid-dynamic runs mentioned in previous chapter V.
Fermentations have been performed on the same products used in experimental tests on lab-scale.

6.1 Experimental apparatus and analytical methods.

Pilot-scale fermentation experiments carried out in a 30L batch reactor describe and developed in previous chapter V.
The fermentation is monitored measuring the following variables:

d) *pH:* pH of the fermented suspension is measured each hour. Three samples of 50 mL of suspension is collected in Falcon vials. A triplicate measurements has been performed using a pHmeter mod. Expandable IonAnalyzer EA920 – Orion Research (Appendix A.1);
e) *Bacterial growth:* samples of the fermented product have been sowed on Petri Plate in order to measure the growth of lactobacilli.
   MRS agar broth has been used. Experimental methods are indicated in Appendix A.2.
f) *Substrate concentration.* Concentration of substrates consumed by lactobacilli

during fermentation is measured by suitable dosage kit.

- In the case of rice starch (suspension rice/water) an enzyme kit has been used. Analytical method according AOAC method 996.11, AACC method 76.13 and ICC standard method No.168 *(Anon, 1987; Karkalis J., 1985; Theander O. and Aman P., 1979; Batey I.L., 1982)*.(Appendix A.3 for further indication).
- For skim milk similar experimental determination has been used, the concentration of Lactose and D-Galactose are analyzed (See Appendix A.4) using a modified AOAC official method 984.15 *(Beutler H.-O., 1988).*

g) *Acids concentration:* Lactic Acid production during fermentation runs has been determined using an HPLC (Appendix A.5) apparatus. Undesired acid compounds as Acetic acid, Butanoic acid, Propionic acid and Ethanol have been determined using GC (Appendix A.6) apparatus.

6.2 Rice flour/water suspension.

Quantities on rice flour suspension supplied at the reactor have been of 30 kg: 10 kg of rice flours are mixed in 20L of water.

6.2.1 Rice flour/water suspension protocol.

Experimental protocol for rice flour/water suspension is characterized by different steps:

1 Sterilization of pilot fermenter using perborate sterilizing solution;

2 Sterilization of rice flour by dry heat at 120°C for 1 h (10 glass bottle filled by about 1kg of rice flour);

3 Water supply in the vessel and successively tyndallised, directly in the vessel, using a specific alternation of heating and cooling as follows:

- Heating at 70°C for 30 min;
- Cooling at 30°C for 30 min;

- Heating at 70°C for 30 min;
- Cooling to fermentation temperature (37°C).

4 Supplying of rice sterilized flour in pilot fermenter using sterile funnel and Bunsen burner, in order to conditionate the microbiological local environment, under stirring at 150 rpm;

5 Supplying of inoculum using Bunsen burner under moderate agitation of 90 rpm;

6 Starting of fermentation *(T=37°C; 90 rpm)*.

6.2.2 Experimental results.

In the following figures are reported the behaviours of ph, CFU/ml, rice starch concentration and acids concentration.

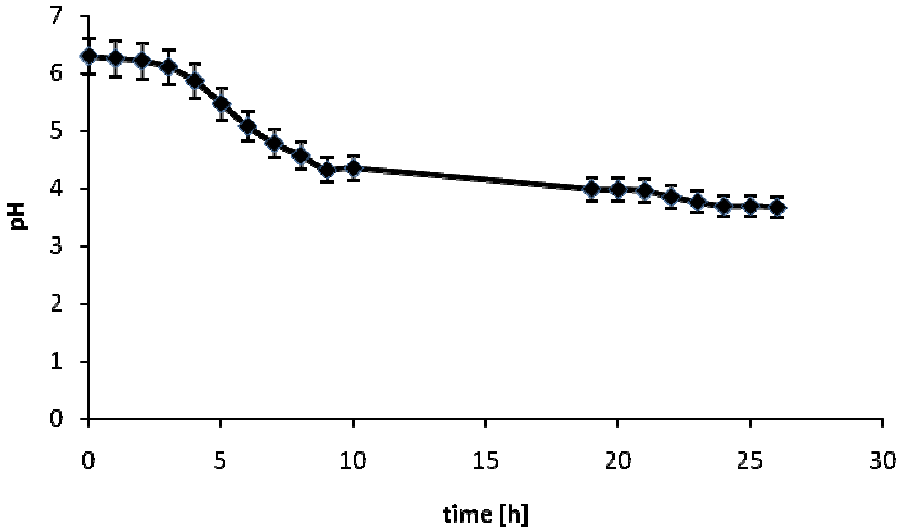


Fig.6.1: pH profile control during fermentation of rice flour/water suspension (T = 37°C, stirrer =90 rpm).

pH measurements show a similar behaviour of lab scale determinations. The value change from 6.4 in first hours of process to 3.5 at the end of 24 h.

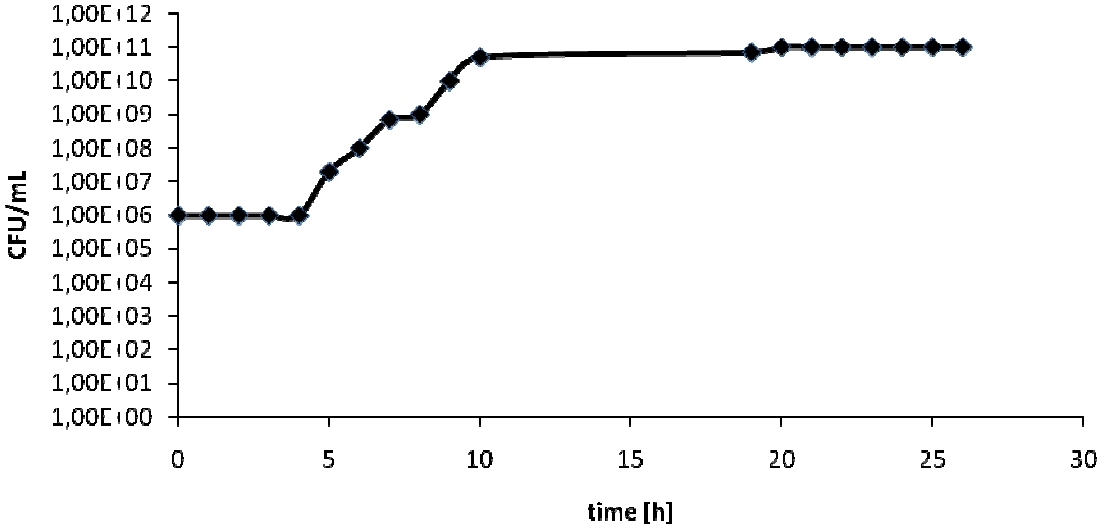


Fig. 6.2: CFU/mL profile during fermentation of rice flour/water suspension(T = 37°C, stirrer = 90 rpm).

In the case of CFU/mL a sensible increase of bacteria counts has been detect compared with laboratory results.

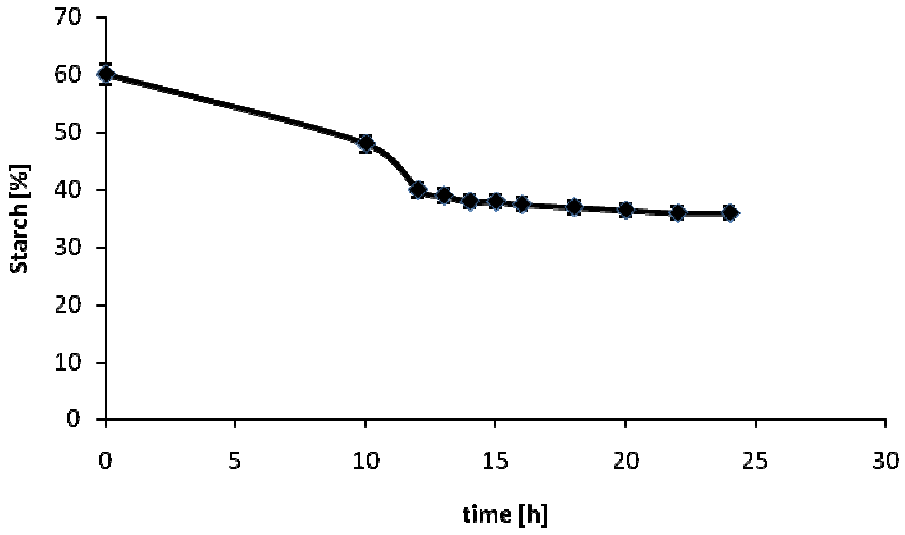


Fig. 6.3: Starch concentration profile during fermentation of rice flour/water suspension (T = 37°C, stirrer = 90 rpm).

For rice flour/water suspension the variations of fermentation substrate are analyzed, in particular starch concentration is reported in fig. 6.3. It is possible to see that before

fermentation starch % is about 60%, while during process it decrease up to 35%.

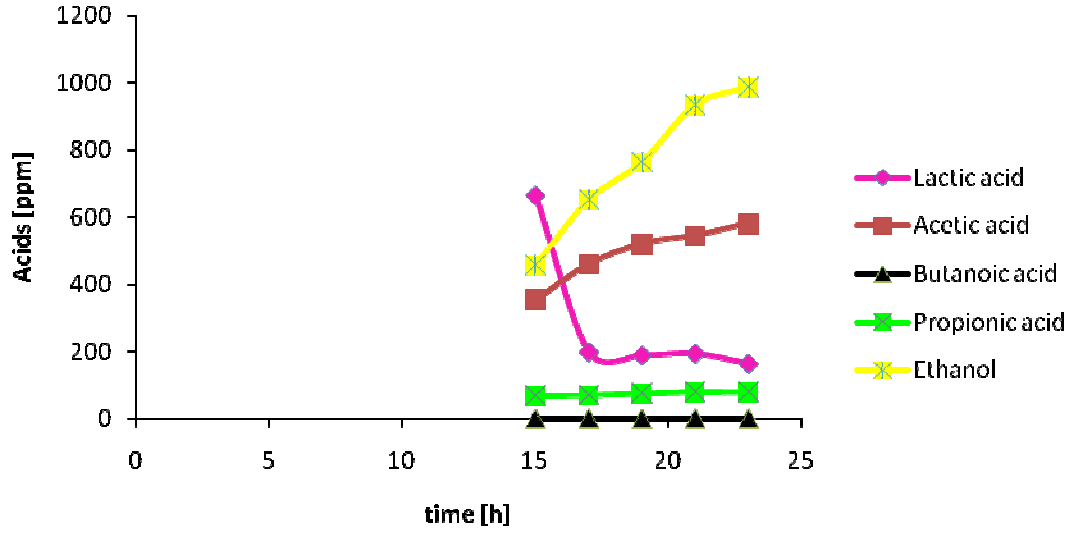


Fig. 6.4: Acids concentration profile during fermentation of rice flour/water suspension (T = 37°C, stirrer = 90 rpm).

Moreover by product analysis a relevant concentration of undesiderable organic acids has been measured with a relatively low and decreasing concentration of lactic acid.

The presence of high concentration of microorganisms measured at the end of fermentation, for rice flour/water suspension, together with the presence of the undesired acid detected led to suppose on the growth of contaminant bacteria, i.e. cocchi or aerobic bacteria. These can be due to incorrect protocol steps used during manipulation of products supplied to reactor.

In order to point out these problems and to quantify the contaminant bacteria, Vancomicin was added to MRS agar using an incubation time of 48 h at 37°C in anaerobic condition to selectively make lactobacilli to growth in Petri plates.

In order to enhance only the growth of lactobacillus.

In parallel, to reveal the growth of undesired bacteria (mainly aerobic bacteria), a selective PCA broth has been used (incubation conditions are 24h and 37°C in aerobic condition).

To enumerate the cocci bacteria MacConkey agar broth has been used (incubation conditions : 24h, 37°C in aerobic condition).

The values of counts of undesired bacteria have been higher than 10^4 CFU/mL.
These results led to point out as critical the step related to supplying of substrate and of the inoculum. The contaminants entrained in the reactor due to manipulation of these products led, at the end of the fermentations, to a consistent presence of undesired bacteria.
In order to prevent this presence and growth, a modification of protocols must be implemented.
In the following paragraph the modified rice suspension protocol is described.

6.2.3 Rice/water suspension.

6.2.3.1 Rice/water suspension protocol.

1. Sterilization of entire plants by means water vapour at 2 atm.
2. Supply of substrates (rice and water).
3. Substrates tyndallisation is obtained directly in the vessel using a specific alternation of heating/cooling steps:
   - Heating at 70°C for 30 min;
   - Cooling at 30°C for 30 min;
   - Heating at 70°C for 30 min;
   - Cooling to fermentation temperature (37°C).
4. Supply inoculum in fermenter using Bunsen burner under moderate agitation of 180 rpm.
5. Start of fermentation *(T=37°C; 180 rpm)*.

6.2.3.2 Experimental results.

In order to take into an account the gelatinization of the starch during tyndallisation of product in the vessel, leading to a difficult of the mixing of the product, the concentration of rice flour has been decreased to 15%.
Using the previous protocol seems to remain a critical point of lower relevance, the

supply of the inoculum. In this case the contaminant entrained in the vessel seem to be much lower if compared to those entrained with the rice.

Using new protocols established by the above considerations experimental runs have been performed on the new substrate (rice/water gel) using comparatively both lab and pilot scale plants.

In the case of lab-scale, pH, CFU/ml and PO_2 are measure. The results are shown in figures 6.5 ÷ 6.7.

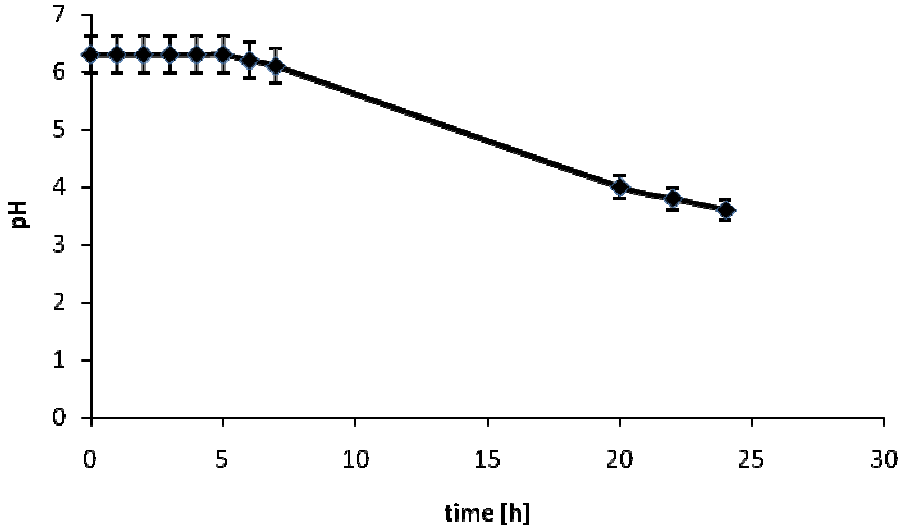


Fig. 6.5: pH profile control during fermentation of rice flour/water gel (T = 37°C, stirrer =150 rpm).

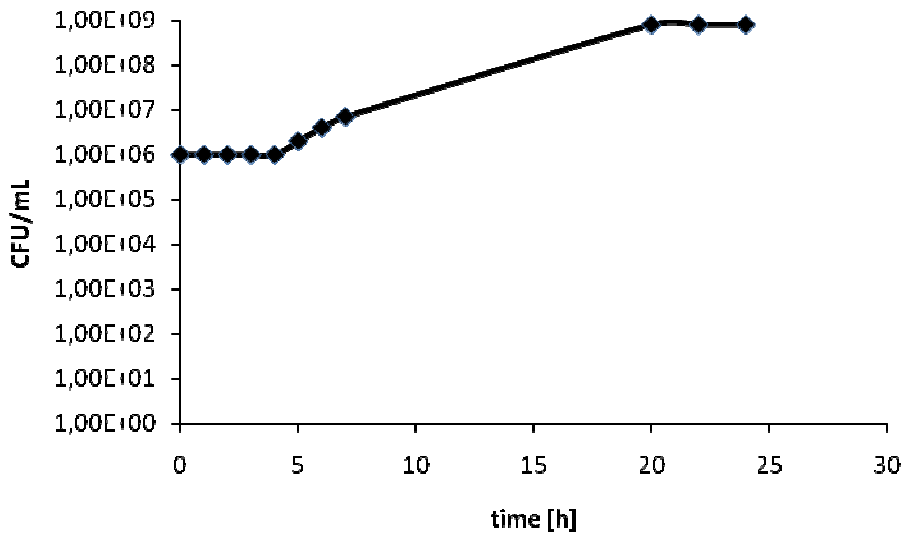


Fig. 6.6: CFU/mL profile during fermentation of rice flour/water gel (T = 37°C, stirrer = 150 rpm).

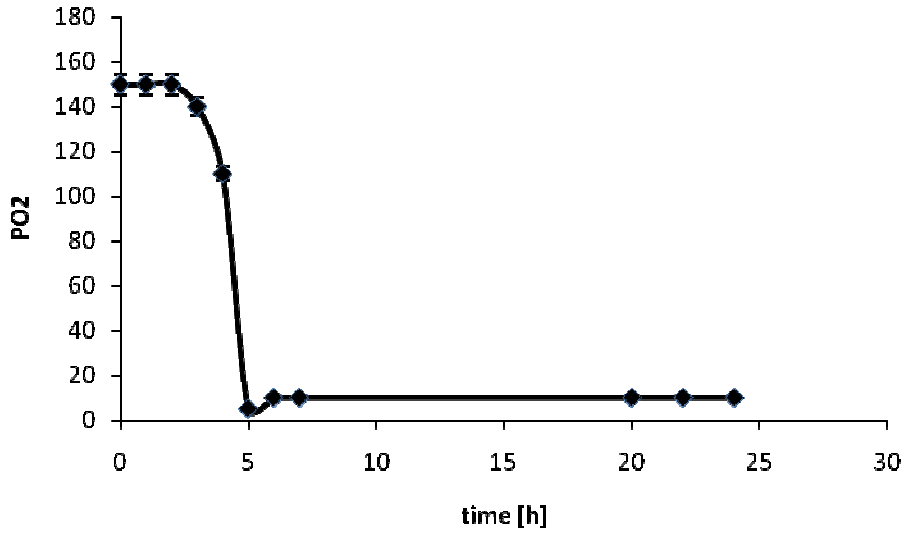


Fig. 6.7: PO_2 profile control during fermentation of rice flour/water gel (T = 37°C, stirrer = 150 rpm).

In the previous figures three main growth phases can be recognized; log phase (0 ÷ 5 h), exponential (5 ÷ 20 h) and stationary (20 ÷ 24 h). This behaviours are connected with low values of partial pressure of O_2 measured from 5 to 24 h.

In the case of pilot-scale pH, CFU/mL, starch concentration and organic acids have

been detected. Results have shown in figures 6.8-6-11.

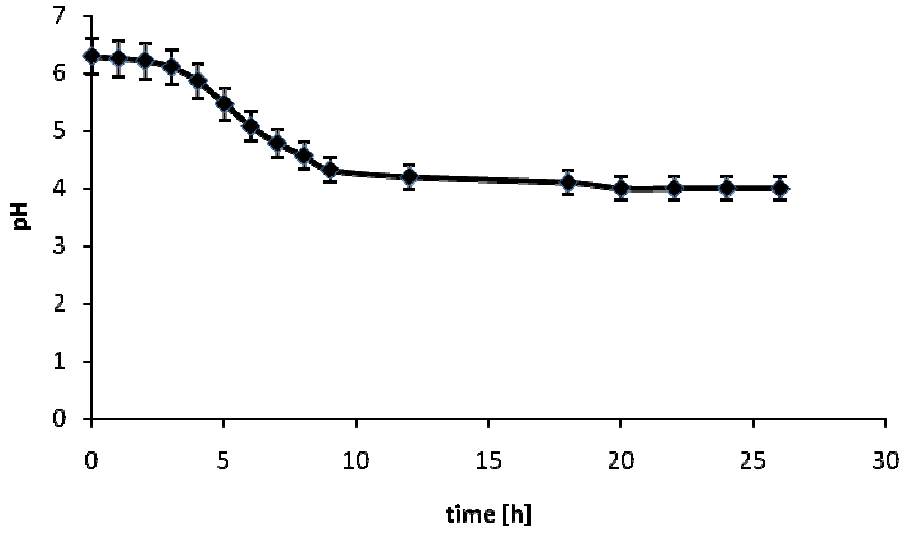


Fig. 6.8: pH profile control during fermentation of rice flour/water gel (T = 37°C, stirrer =180 rpm).

pH measurements in pilot-scale report the same behaviour to lab-scale.

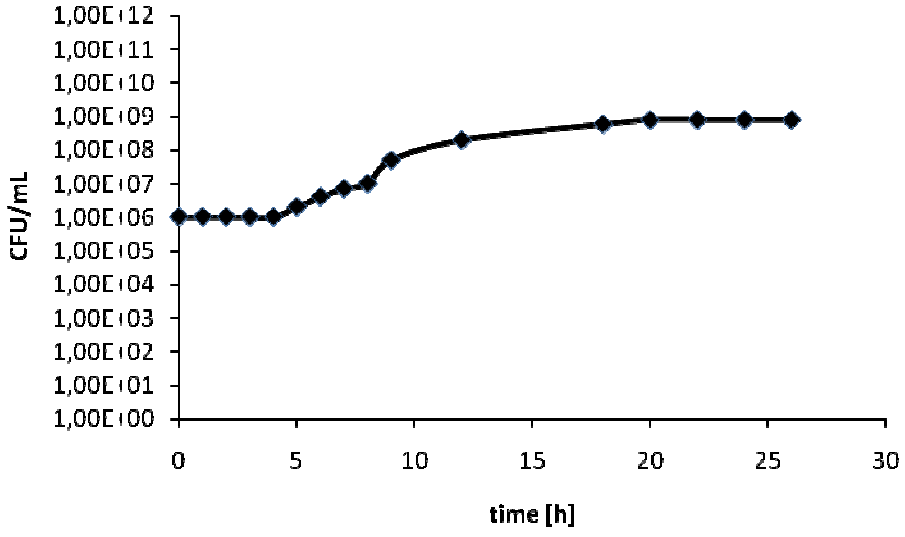


Fig. 6.9: CFU/mL profile during fermentation of rice flour/water gel (T = 37°C, stirrer = 180 rpm).

For new substrate CFU/mL behaviour in pilot-scale it is similar to lab results.

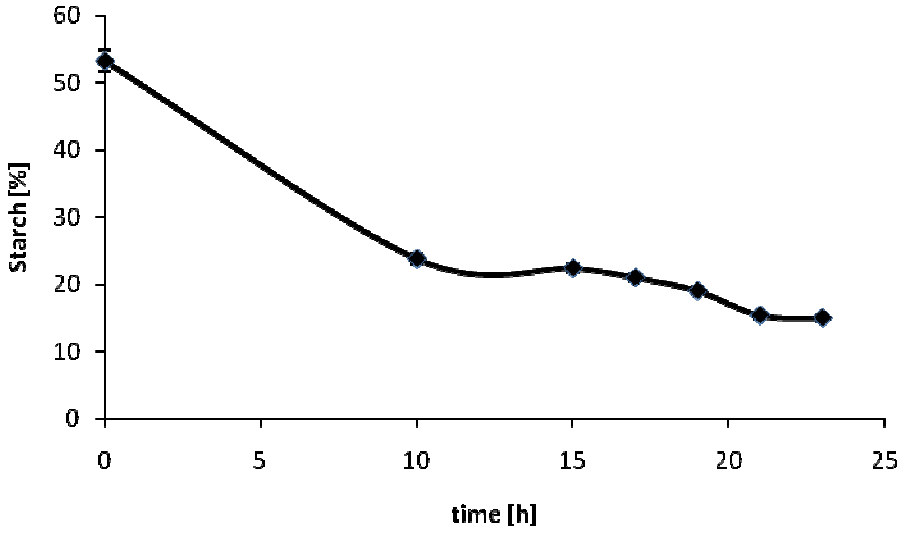


Fig. 6.10: Starch concentration profile during fermentation of rice flour/water gel (T = 37°C, stirrer = 180 rpm).

By analyses of substrate behaviour it is possible see that starch % change from 54% to 18%, this variation justify the good fermentation.

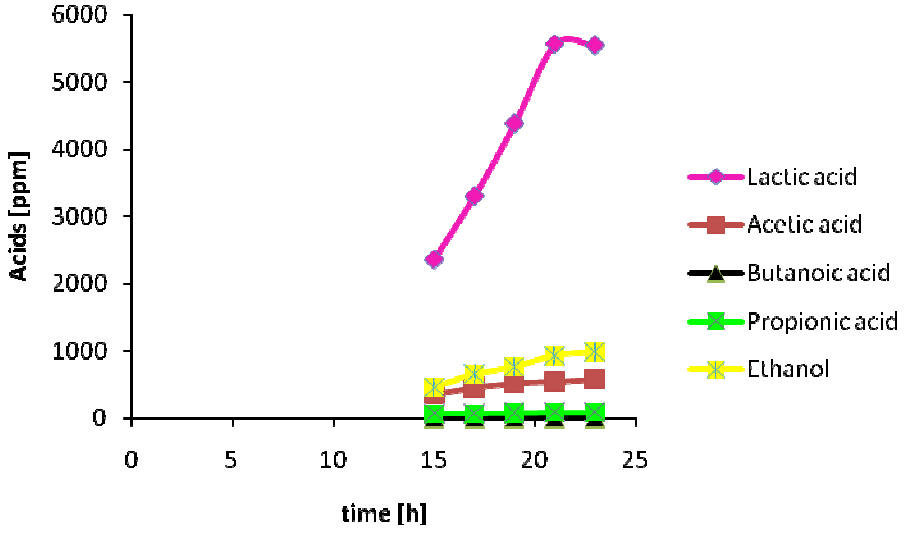


Fig. 6.11: Acids concentration profile during fermentation of rice flour/water gel (T = 37°C, stirrer = 180 rpm).

Results obtained in pilot-scale are the same of similar variables measured in lab scale fermenter. In these cases the contaminants are low (CFU/mL $\leq 10^4$).
Moreover the concentration of lactic acid is sensibly higher of undesired acids.
Results obtained with modified protocol are acceptable, so in order to have a stable products, the fermented suspension must be dehydrated using the previously mentioned spray-drying method.
In fact, solid concentration and the low viscosity of the product can design a direct interface of the pilot plant to a spray drying process.

6.2.3.3 Kinetic models for batch cultures in rice/water gel fermentation.

The microorganisms growth kinetics is represent by Monod model that takes into account the mass-conservation condition linking substrate uptake to biomass formation and the dependence of the specific growth rate on limiting substrate concentration.
The equation of Monod kinetics utilized in this study is following reported:

$$\mu_g = \frac{\mu_m S}{K_s + S} \qquad (1)$$

The expression utilized don't present death growth because the fermentation process is stopped at stationary phase to have maximum microorganisms concentration and ignore substrate inhibition because the high reaction volume became negligible inhibition.
To describe kinetics evolution of fermentation process experimental determination of growth parameters K_s and μ_m are necessary.
The parameter μ_m, maximal specific growth rate, has very lucid biological meaning: it is upper limit of μ variation on specified nutrient medium. It could not be attained in reality because of its asymptotic nature: $\mu \rightarrow \mu_m$ as $S \rightarrow \infty$. However, in practice μ_m is achieved if $S \gg K_S$. It should be remember that μ_m is not absolute maximum of growth rate, because it depends on the nature of the limiting substrate.
Saturation constant K_S could be defined functionally such as concentration of limiting

substrate that provides a specific growth rate equal to 0.5 of μ_m. K_S is a measure cell affinity to substrate: the lower is K_S the better the organism is adapted to consume substrate from dilute solution.

To determine numeric values of K_S and μ_m the definition of doubling time is used such as known of t_{exp} (exponential phase duration) and substrate concentration at 0.5 of μ_m.

Doubling time, t_d, is defined as the time required for biomass to be doubled and assuming a constant maximum value of specific growth rate this will be:

$$t_d = \ln 2/ \mu_m \qquad (2)$$

Data of microorganisms growth obtained in experimental tests (pilot scale) have been used to calculate kinetics parameters in the case of the rice/water gel that represent the only optimal substrate. They are:

$\mu_m = 0.04\ h^{-1}$

$k_S = S\ \mu_m = 23$ g/L.

6.2.3.4 Process productivity for rice/water gel fermentation.

To complete kinetics study process productivity must be calculate.

The productivity of a batch culture is given by final concentration of whatever is being made divided by the complete time of the batch operation. This time period includes the running of the bioreactor. The unit of productivity, which is usually denoted by P, are kg product $m^{-3}\ h^{-1}$.

To calculate the correspondence from CFU/mL and kg/mL of lactobacillus of interest different experiment are doing:

- Lactobacillus inoculums are supply in 200 mL of culture broth (MRS) to growth at 37°C.
- After 48h of incubation count plates are doing.
- All culture broth, in which microorganisms are growth, are to whirl in a centrifuge in aliquots of 20 mL and at stirring velocity of 4000 rpm.

- The centrifuged are separate in supernatant and solid phase.
- All solid phases are weighted.

To assure the accuracy, reliability, and reproducibility of the collected data, all tests were performed in triplicate and the result are reported following:

$CFU/L = 1.4*10^{-12}$ kg/L

By this results if assumed that total time period of process for rice flour/water gel is about 30 h, it possible calculate productivity values that are:

$P = 0.0093$ kg/$m^{-3}h^{-1}$

6.3 Skim milk.

6.3.1 Skim milk protocol.

Experimental protocol for skim milk (commercial in 1L tetrapack brick) is characterized by this steps:

4 Sterilization of fermenter by perborate sterilizing solution;

5 Supplying of 20L of milk using sterile funnel and Bunsen burner;

6 Milk is tyndallised directly in the vessel using a specific alternation of heating/cooling steps:
   - Heating at 70°C for 30 min;
   - Cooling at 30°C for 30 min;
   - Heating at 70°C for 30 min;
   - Cooling to fermentation temperature (37°C).

7 Supplying of inoculum using Bunsen burner under moderate agitation of 90 rpm;

8 Fermentation start *(T=37°C; 90 rpm)*.

6.3.2 Experimental results.

In the following figures are reported the behaviours of ph, CFU/mL, Lactose and Lactose + D-Galactose concentration and acids concentration.

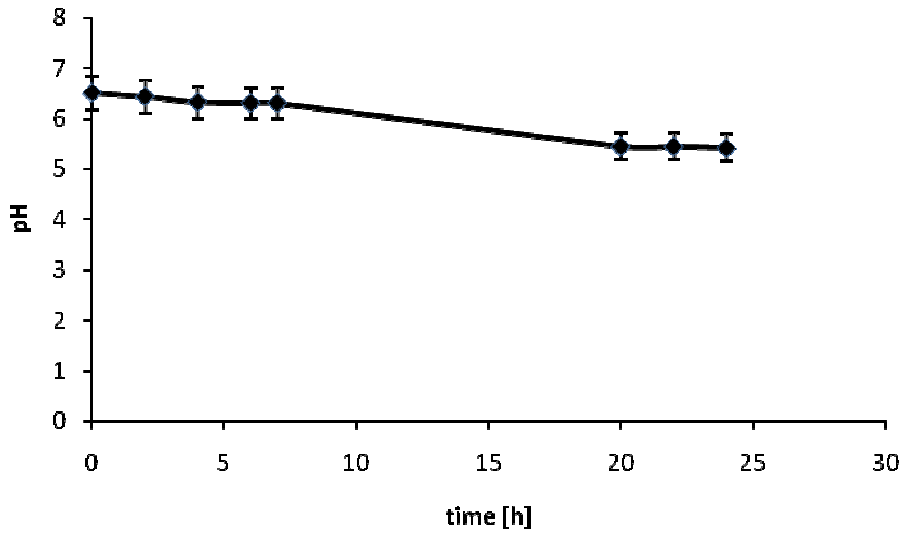


Fig. 6.12: pH profile control during fermentation of skim milk (T = 37°C, stirrer =90 rpm).

In skim milk fermentation pH measurements show a similar behaviour of lab scale determinations.

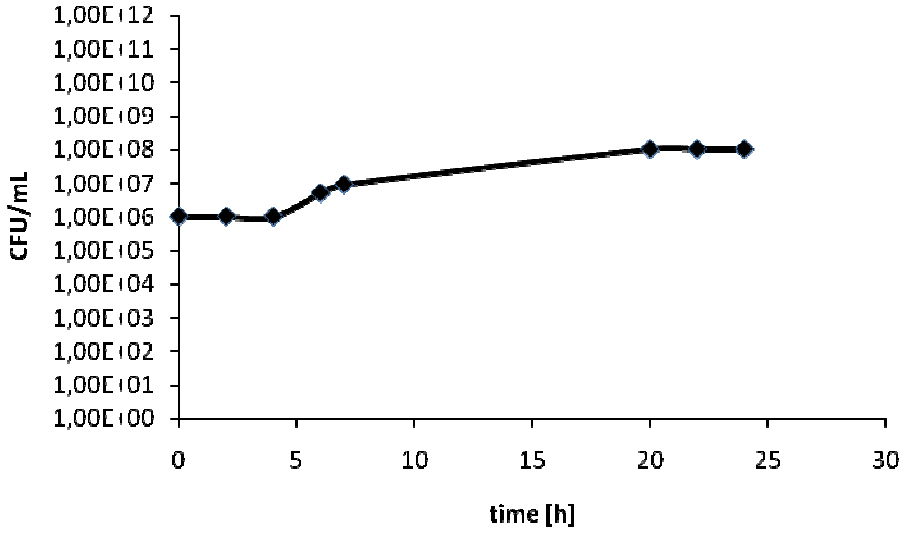


Fig. 6.13: CFU/mL profile during fermentation of skim milk (T = 37°C, stirrer = 90 rpm).

CFU/mL measurements show a similar behaviour of lab scale determination.

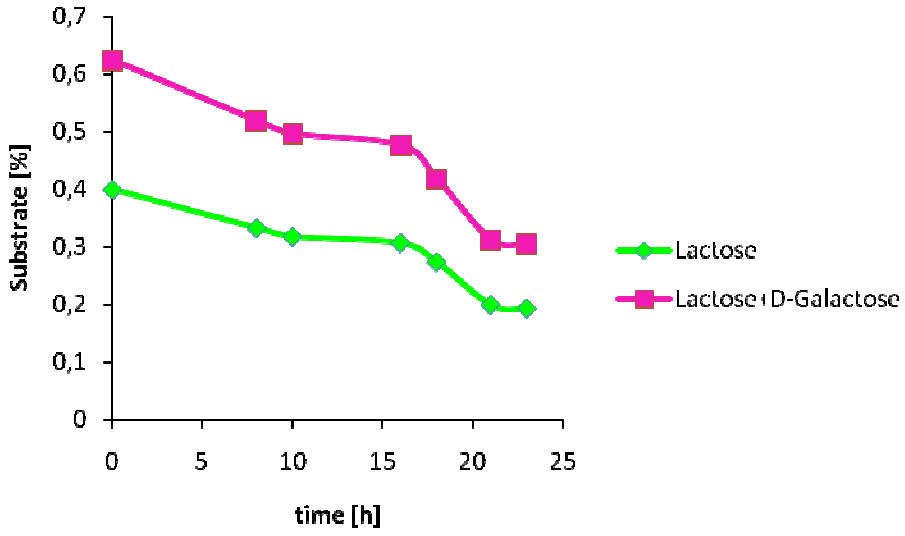


Fig. 6.14: Lactose and Lactose + D-Galactose concentration profile during fermentation of skim milk (T = 37°C, stirrer = 90 rpm).

Analyses of substrate behaviour for skim milk is about Lactose and Lactose + D-Galactose concentration profile.

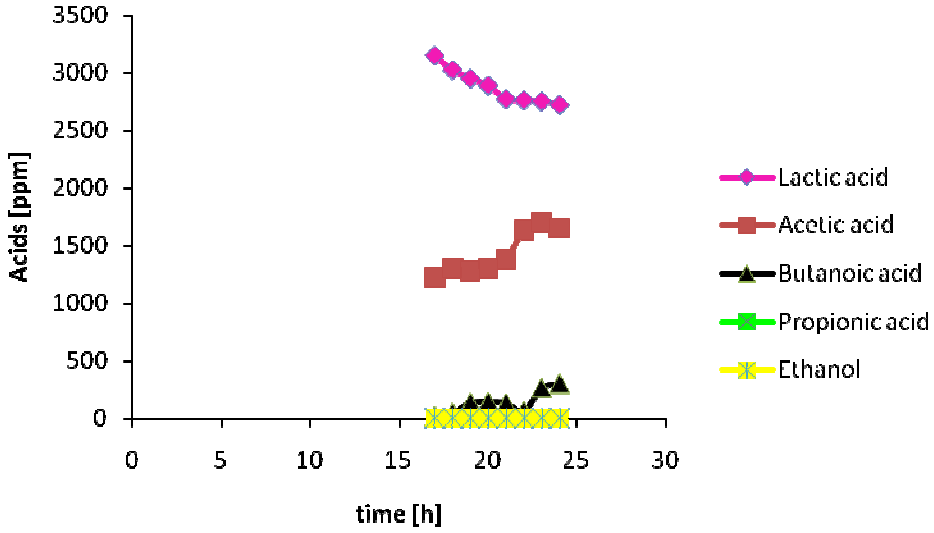


Fig. 6.15: Acids concentration profile during fermentation of skim milk (T = 37°C, stirrer = 90 rpm).

Moreover, during skim milk fermentation, a relevant concentration of undesiderable organic acids, such as Acetic acid and Butanoic acid, has been measured.

The presence of the undesired acid detected led to suppose on the growth of contaminant bacteria, i.e. cocchi or aerobic bacteria. These can be due to incorrect protocol steps used during manipulation of products supplied to reactor.

In order to point out these problems and to quantify the contaminant bacteria, Vancomicin was added to MRS agar using an incubation time of 48h at 37°C in anaerobic condition to selectively make lactobacilli to growth in Petri plates.

In order to enhance only the growth of lactobacillus.

In parallel, to reveal the growth of undesired bacteria (mainly aerobic bacteria), a selective PCA broth has been used (incubation conditions are 24h and 37°C in aerobic condition).

To enumerate the cocci bacteria MacConkey agar broth has been used (incubation conditions : 24h, 37°C in aerobic condition).

The values of counts of undesired bacteria have been higher than 10^4 CFU/mL.

The contamination can be due to the presence of other contaminant different from mentioned bacteria. To verify these problems, milk must be treated with a modificate protocol that is described in following paragraph.

6.3.2.1 New skim milk protocol.

1 Sterilization of entire plants by means water vapour at 2 atm.
2 Supply of milk.
3 Substrates tyndallisation is obtained directly in the vessel using a specific alternation of heating/cooling steps:
   - Heating at 70°C for 30 min;
   - Cooling at 30°C for 30 min;
   - Heating at 70°C for 30 min;
   - Cooling to fermentation temperature (37°C).
6 Supply inoculum in fermenter using Bunsen burner under moderate agitation of 90 rpm;
7 Start of fermentation *(T=37°C; 90 rpm)*;

The change of the above protocol seems to be connected to an higher initial contamination of the plant that tyndallisation process does not eliminate. To solve these problems, milk must be treated in the vessel using a UHT cycle, that cannot be implemented in the present fermenter model.

Chapter VII

Drying of fermented products on a pilot-scale

Relating to experimental results shown in previous chapter, only rice/water gel is fed to dehydration process. Between drying technology spray dryer is utilizes; it is rarely cost competitive with two-step dewatering and solid-drying processes.

Spray-drying can be used to produce large amounts of food ingredients relatively inexpensively, the spray-dried powder can be transported at a low cost and can be stored in a stable form for prolonged periods.

This drying process is an economical one for preparing industrial scale quantities of products even containing probiotic microorganisms. Its application to generate preparation of Lactobacillus has recently received considerably interest. This spray-dried powders may be applicable to downstream processes, adjunct for dried pasta production, bakery production, etc., exploiting not only the residual viable microorganisms, but also the non-viable bacteria that can have a strong probiotic effect and a considerably modulation of the host's immunity response, so increasing the natural resistance to invading pathogens.

The relatively higher complexity of spray-drying apparatus in terms of droplets generation, powder capture linked to size of the solid, can be justified on the basis of some improvement in product quality, such as particle form, size, product flavor, color or heat stability.

7.1 Spray Dryer set-up.

In the following figure 7.1 plot of the pilot-scale spray dryer is shown.
Drying chamber (V = 2500-3000 L), powder capture device (cyclone) can be seen together with instrumentation for measures and controls of drying process.

Fig. 7.1: Pilot plant: Spray dryer.

Its operative range are:
20 < T < 250°C
2 < Feed < 15 L/h
Max $\%_{SOLID}$ = 20%.
Spray-dryer plant includes an atomizer to disperse the feed into the gas stream. The hot gas, obtained by use of methane combustion and heat exchanger, inlet to the dryer is on the top (fig.7.2) and the gas distribution within the chamber is based on vane ring. The

gas outlet is at opposite end of the chamber from which feed is introduced. The vessel is equipped for separation of the product by gravity.

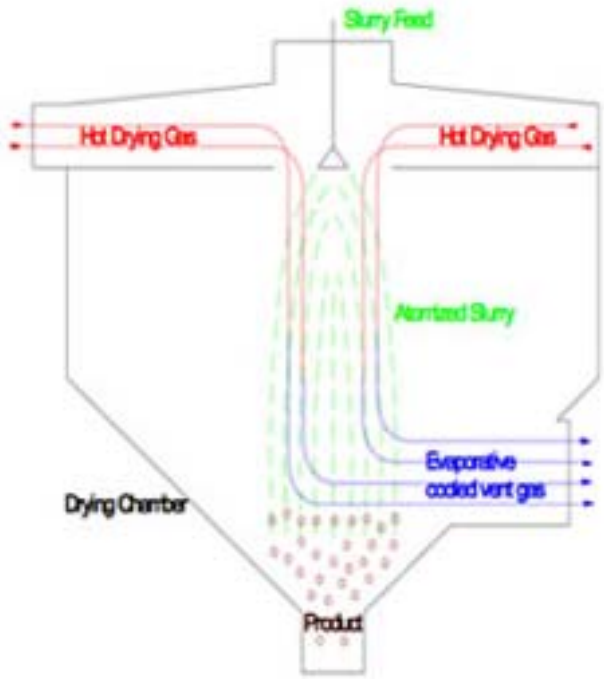


Fig. 7.2: Spray dryer configuration.

Airflow patterns depend on chamber design and define airflow. Atomization and evaporation create complex localized flow patterns around the atomizer. These patterns contain some circular flow components. In mixed flow dryers, a portion of the air is supplied tangentially. Also, cooling air is introduced at the exit, to protect a sensitive product.

Product flow is co-current to gas flow, the dryer is designed to aid particle separation by centrifugal forces.

The atomizer is a high-speed rotating disc (Fig. 7.3), it's function is to break the feed material into many droplets to increase the surface area and to form the droplets that will result in the desired particle size and shape for final product. Flow rate and droplet size are affected by fluid pressure.

Fig7.3: Atomizer.

The gas stream is heated before entering the dryer cylinder; this gas stream provides the thermal energy needed to:

1. Heat the material to be dried.
2. Heat and evaporate the liquid and heat the vapor to the exhaust temperature.
3. Compensate for conduction, convection, and radiation heat losses from the chamber.
4. Provide the energy required for the heat of reaction, if applicable.
5. Provide the energy required for heat of crystallization, if applicable.
6. Avoid conditions where vapor might condense in the exit stream.

After leaving the drying chamber, all gases and vapor from drying usually pass through product/dust recovery equipment before being released to atmosphere. Cyclonic separator are used (Fig. 7.4) for product recovery.

Fig.7.4: Cyclonic separator.

Fans induce the gas flow through the heater, drying chamber, and product/dust collection equipment. In the plant there is the most simple fan capability to accommodate the pressure-drop through the inlet gas filter, gas heater, gas inlet duct, and dust collector, which typically amounts to 3.75 to 12.5 kPa (15-50 inches of water).

7.2 Dehydration experimental runs.

Fermented rice/water gel is fed to spray dryer and dehydrated using suitable process conditions in order to obtain a dry functional constituent to add to baby foods.
Process condition used in runs are:

Vrot = 30.000 rpm (atomizer speed) (Drop size = 150 µm)

Feed = 15 kg/h

T_{IN} *= 180-190 °C*

T_{OUT} *= 80-90 °C.*

Collected dry solid has good brightness and is characterized by a mean size of 40 μm. This solid shows a good flowability and a low stickiness.

The presence of viable and total (viable and non-viable) microorganisms has been verified on the dried solid. In the first case a suitable amount of powder is dispersed in physiological solution and after a emerging agitation is sowed on Petri plates according to previously laboratory methods mentioned, the growth of live Lactobacilli.

In the second case PCR permit to evaluate the fraction of death and live lactobacilli.

Result obtained, confirm that PCR count was of 10^9 CFU/mL, while viable microorganisms was very low and lower than 10^4. This values is indicative that the drying process, i.e. the outlet temperature of the flow is too high to obtain high concentration of viable lactobacilli.

Chapter VIII

Development of a fermentative reactor on industrial scale.

In order to scale-up the fermentation/drying process to a pre-industrial scale must be defined the following items:

1. General recommendations of the pre-industrial plant;
2. Asses the aim of the plant;
3. Assessing of main operative units;
4. Asses protocol of the fermentation process;
5. Point out process critical points.

8.1 General recommendations of the pre-industrial plant.

Project, realization and setting up of industrial plant must respect law regulation as recommend to GMP (Good Manufacturing Practice).
Electric equipment adopted must be coherent to EEC directives, in particular:

- Dir. 98/37/CE - Mechanical Directive.
- Dir. 94/9/CE - Atex Directive.
- Dir. 89/336/CE - Electromagnetic Directive.

- Dir. 73/23/CE - Low voltage Directive.

Materials and equipments must be coherent to IEC and/or CEI laws, they must have IMQ mark.
All equipment that is at high pressure must coherent to directive 97/23/CE (PED).

8.2 Aim of the plant.

Aim is project an food 300L reactor to fermentation of powder substrates by microbiological strains.
The fermentation must be of batch type equipped by thermal and mixing systems.
Fundamental is system aseptic to avoid product contamination.
System characteristics must allow of:

- Substrate sterilization at 100°C for 60 min;
- Reactor sterilization by water stream for 20 min;
- Water pasteurization at 80°C for 30 min;
- Cooling pasteurized water;
- pH analyses from 3 and 7;
- Air output from fermenter by CO_2 input;
- Temperature control from 20 to 50°C;
- Automatic cover movement;
- Automatic substrate powders movement;
- Automatic reactor clean by water stream.

8.3 Main operative units.

Coherently with plant necessary characteristics, the system is composed by (fig.8.1):

1. Batch reactor;
2. Cereal powder supply;

3. Heated hopper;
4. Input CO_2 equipment;
5. Temperature control equipment;
6. pH control equipment;
7. Automatic reactor clean equipment;
8. PLC to system control.

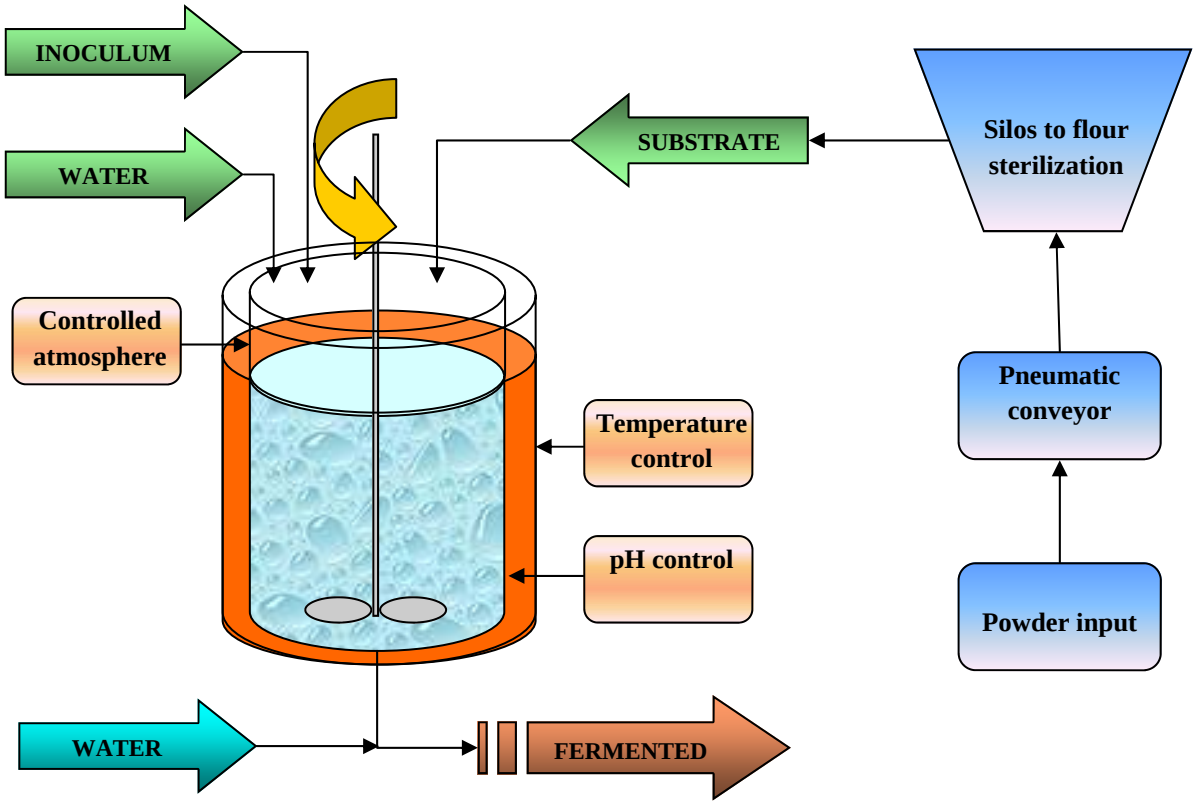


Fig. 8.1: Schematic plan.

8.3.1 Batch reactor

A suitable volume of the reactor is of 500L in a cylindrical shape (D = 80 cm and H = 100 cm).

The following recommendation must be guaranteed:

- Mixing is based on one or more impellers; impeller velocity must be controlled by an inverter system.
- Driven motor of the impellers is mounted on reactor cover that is equipped by an automatic open system.
- Baffles must located in a reactor in order to assure a complete mixing of the suspension.
- Reactor must be equipped by the following automatic units:
    1. Water supply;
    2. Cereal powder supply;
    3. Inoculum supply;
    4. CIP/SIP unit for automatic washing/sterilization of the equipment;
    5. Fermented product output;
    6. Water discharged output top plan cleaning;
    7. N°2 Temperature probes;
    8. N°1 pH probe;
    9. pH correction unit;
    10. CO_2 supplying system conditioning the reactor freeboard;
    11. A temperature control system;
    12. Sampling ports;
    13. PLC unit;
    14. A security control.

8.3.2 Cereal powder supply.

This system is formed by two apparatus:

1. *Pneumatic conveyor*.

    This unit has the aim of supply the powdered cereal to the heated hopper. This apparatus, by mean of a section system based on a venture tube, transport cereal flour from industrial bags to the heated hopper.

2. *Heated hopper.*

   This hopper has the aim of reduce to minimum of the undesired microorganisms level using heat treatment. Treatment temperature must be in the range 90÷120°C.

8.4 Process Protocol.

According to protocol developed in previous chapters a schematic description of main process steps:

1. *Weigh of substrate.*

   Solid substrate stored in the funnel is weighed using electronic loading cell interfaced to an automatic computerized system.
2. *Substrate pasteurization.*

   The preselected amount of solid is fed to heater hopper.
3. *Reactor washing and sterilization.*

   CIP/SIP unit.
4. *Water pasteurization.*

   Process water, to be mixed to pasteurized solid, is pasteurized at a temperature of 80°C and then cooled at ambient temperature and supplied to the reactor.
5. *Supply of substrate.*

   Substrate is supplied to the reactor vessel and mixed to the water. Temperature of the suspension is controlled to a 37°C.
6. *Supply of inoculums.*

   The inoculum is supplied to reactor.
7. *Start of fermentation.*
8. *CO_2 supply.*

   Air entrained in reactor during filling processes, is eliminated by using flowing CO_2 under moderate agitation.

9. *pH control.*

   pH is monitored on-line in order to control the status of fermentation. The fermentation process can be controlled by using a suitable alkaline solution in order to avoid that low value of pH can led to a stop of fermentation.

9. *Product discharge at the end of fermentation.*

   At the end of fermentation, the fermented solution is discharged and directly fed to spray drying for dehydration after an optional dilution process.

10. *Plant clean.*

    Plant clean using a C.I.P. procedure.

8.5 Plant plan.

In fig. 8.2 it is reported a scheme of the pre-industrial plant as designed following these experimental studies and that will be implemented in Plasmon Pilot Plant of Ozzano Taro.

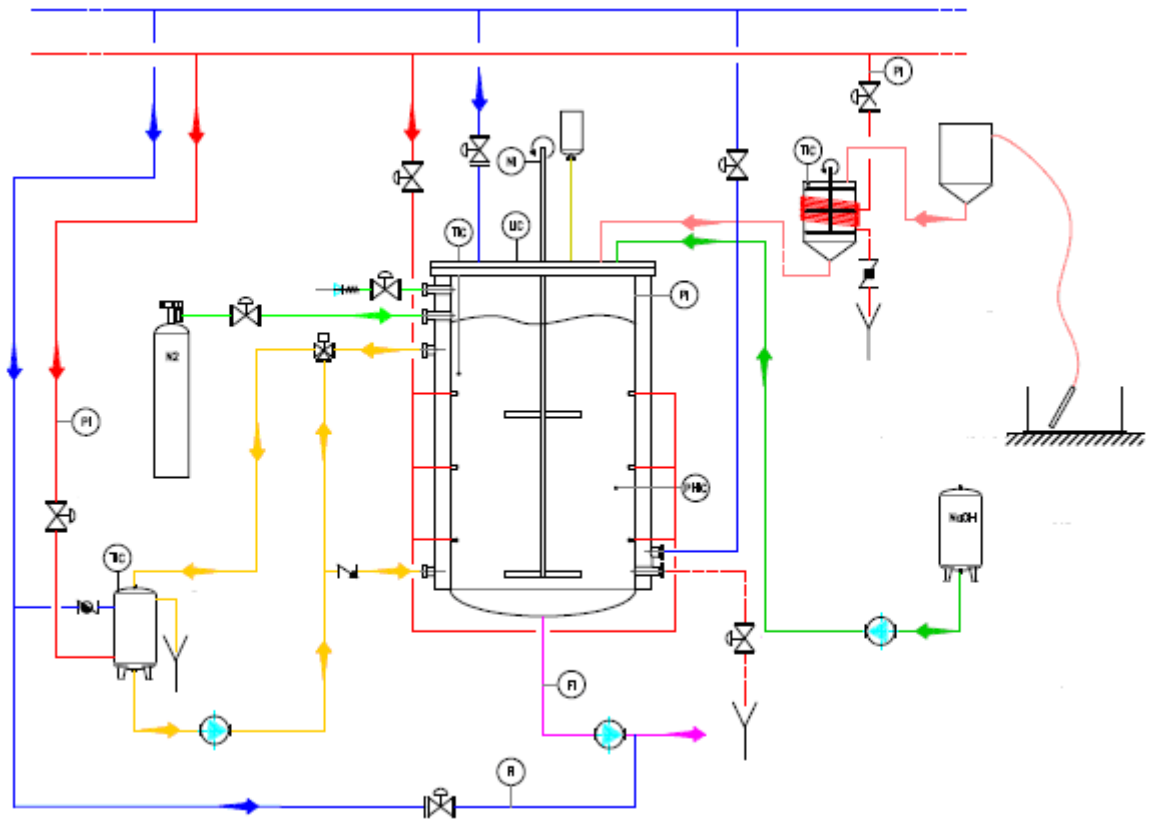


Fig. 8.2: Industrial plant project.

Chapter IX

Conclusions.

Objective of this work is to study the fermentation process as a valuable process to produce ingredients of functional foods using dried cereals and milk based.

Fermentation of raw cereal products (rice, wheat, oats) as suspension followed by a mild spray-drying processes can be useful to obtain a powered components with probiotic/prebiotic effect even if involved in subsequent treatment processes, as cooking, kneading etc..

Fermentation of selected substrates has been performed using a Lactobacillus paracasei named 10/2 or CBA-L74 where performances was pointed out and optimized in lab scale tests. This particular Lactobacillus, selected from baby faeces, has shown a good growth performances for both substrates with bacterial concentration much higher than that requested for lives of probiotic standards.

Cereals and skim milk fermented at lab scale showed a good taste and flavour.

Main results of cereal (rice) and milk fermentations are shown in the following table 9.1.

Substrates	pH	PO2 [%]	CFU/mL
Rice flour/water suspension	4	0	10^8 - 10^9
Skim milk	5.8	12.5	10^8

Tab.9.1: Laboratory experimental results.

The lab-scale protocol has been tested using a fermenter (30 L), pointing out critical points related, essentially, to solid handlying and supply to the fermentation vessel.
Experimental tests performed in this scale shown the need of performing the fermentation using a dedicated protocol able to eliminate the contaminations due to undesired microorganisms (i.e. cocci, aerobic) that led to a sensible presence of undesired organic acid (tab.9.2).

Substrates	Rpm	CFU/mL (24hr)	pH (24hr)	Substrate variation	Lactic Acid increase	Acetic acid increase	Butanoic acid increase	Propionic acid increase	Ethanol increase
Rice flour/water suspension	90	10^{11}	3.5	Starch 60%÷35%	< 50 ppm	≈600 ppm	< 50 ppm	≈90 ppm	≈1000 ppm
Skim milk	90	10^8	5.5	Lactose + D-Galactose 0.63%÷0.3%	≈2800 ppm	≈1600 ppm	≈400 ppm	< 50 ppm	< 50 ppm

Tab.9.2: Pilot experimental results.

A new protocol has been developed in order to overcome contamination.

In this practical a stronger pasteurization (Tyndallisation) has be applied an a more diluted suspension in order to avoid gelification of rice/water suspension due to a rice starch.

In the following table 9.3 experimental results are shown.

Substrates	*Rpm*	*CFU/mL (24hr)*	*pH (24hr)*	*Substrate variation*	*Lactic Acid increase*	*Acetic acid increase*	*Butanoic acid increase*	*Propionic acid increase*	*Ethanol increase*
Rice flour/water gel	*180*	*10^9*	*3.5*	*Starch 60%÷18%*	*≈5800 ppm*	*≈500 ppm*	*< 50 ppm*	*<50 ppm*	*≈600 ppm*

Tab.9.3: Pilot experimental results for rice/water gel.

Remaining critical point with lower relevance is the supply of the inoculum but the irrelevant quantity of the inoculums lead to neglect this critical point.

New protocol has been developed to skim milk. No different results, compared to previous protocol, have been observed.

For rice/water gel suspension fermentation runs kinetics parameters, assuming a Monod kinetic regime, and fermentation productivity have been calculated:

$\mu_m = 0.04\ h^{-1}$

$k_S = S_{\mu m} = 23\ g/l.$

$P = 0.0093\ kg/m^{-3}h^{-1}$

Fermented product has been dehydrated by a pilot scale spray drying using the following main process conditions:

Vrot = 30.000 rpm (atomizer speed) (Drop size = 150 µm)

Feed = 15 kg/h

T_{IN} = 180-190 °C

T_{OUT} = *80-90 °C.*

Probiotic/prebiotic powder has been obtained with a main size of 40 µm.

This solid shows a good flowability and a low stickiness.

The presence of viable and total (viable and non-viable) microorganisms has been verified on the dried solid. In the first case a suitable amount of powder is dispersed in physiological solution and after an emerging agitation is sowed on Petri plates according to previously laboratory methods mentioned, the growth of live Lactobacilli.

In the second case PCR permit to evaluate the fraction of death and live lactobacilli.

Result obtained, confirm that PCR count was of 10^9 CFU/mL, while viable microorganisms was very low and lower than 10^4. This values is indicative that the drying process, i.e. the outlet temperature of the flow is too high to obtain high concentration of viable lactobacilli.

The experimental tests performed led to the design of a plant at higher scale with a reactor vessel of about 300L.

Functional powder obtained must to be tested in-vitro and in-vivo procedures in order to confirm the probiotic/prebiotic effect on the host and moreover confirming the effect on immunological response.

Appendix

A.1 pH-meter.

A pH meter is an electronic instrument used to measure the pH (acidity or alkalinity) of a liquid (though special probes are sometimes used to measure the pH of semi-solid substances). A typical pH meter consists of a special measuring probe (a glass electrode) connected to an electronic meter that measures and displays the pH reading.

A.1.1 The probe.

The pH probe measures pH as the activity of hydrogen ions surrounding a thin-walled glass bulb at its tip. The probe produces a small voltage (about 0.06 volt per pH unit) that is measured and displayed as pH units by the meter. For more information about pH probes, see glass electrode.

A.1.2 The meter.

The meter circuit is no more than a voltmeter that displays measurements in pH units instead of volts. The input impedance of the meter must be very high because of the high resistance — approximately 20 to 1000 MΩ — of the glass electrode probes typically used with pH meters. The circuit of a simple pH meter usually consists of operational amplifiers in an inverting configuration, with a total voltage gain of about -17. The inverting amplifier converts the small voltage produced by the probe (+0.059 volt/pH) into pH units, which are then offset by seven volts to give a reading on the pH scale.

A.2 Plate count.

Plate count method has traditionally been used for determination of viability of bacteria, but there are obvious disadvantages. First, plate count requires long incubation times. Plate count method is often hampered by technical difficulties such as clumping and inhibition by neighbouring cells. The choose of enumeration medium and incubation conditions for specific species may also be challenging.

Direct plate counting is a method used to count the number of viable cells in a sample. Once the cells to be counted have been isolated, they are to be diluted (fig. A.2-1) due to the fact that too many cells will cause the Petri plate to be so densely populated with colonies, that they would be impossible to count. After the cells have been diluted, they are incubated on an agar medium until colonies form. It is at this time that the cells may be counted.

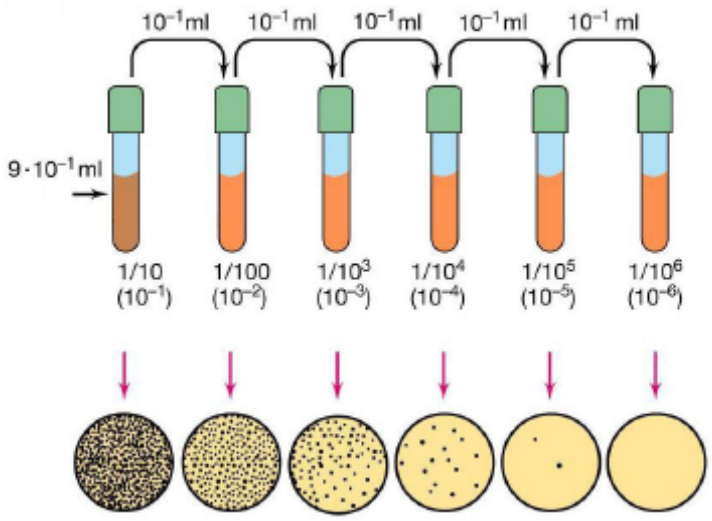


Fig.A.2-1: Dilution system.

There are two main methods of direct plate counting: spread plate method and pour plate method.

The spread plate method, that is used in the experimental analyses, consists of evenly spreading the diluted sample over an agar plate (fig. A.2-2). When using this method, a volume of 0.1 ml of the diluted sample should not be used since the agar will not be able to absorb the excess. Using this method yields colonies that form on the surface of the agar.

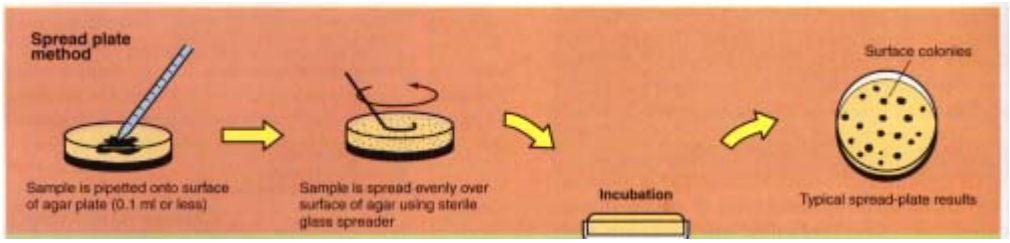


Fig.A.2-2: Scheme of spread plate method.

When using the pour plate method (fig. A.2-3), a diluted sample is pipetted into a sterile Petri plate, then melted agar is poured in and mixed with the sample. Using this method allows for a larger volume of the diluted sample. Usually in the range of 0.1 - 1.0 ml. This method yields colonies that form colonies throughout the agar, not just on the surface. Caution must be taken with this method to ensure that the organism to be counted can withstand the temperatures associated with the melted agar.

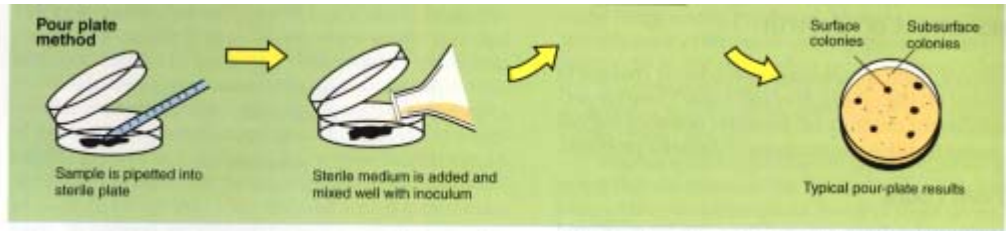
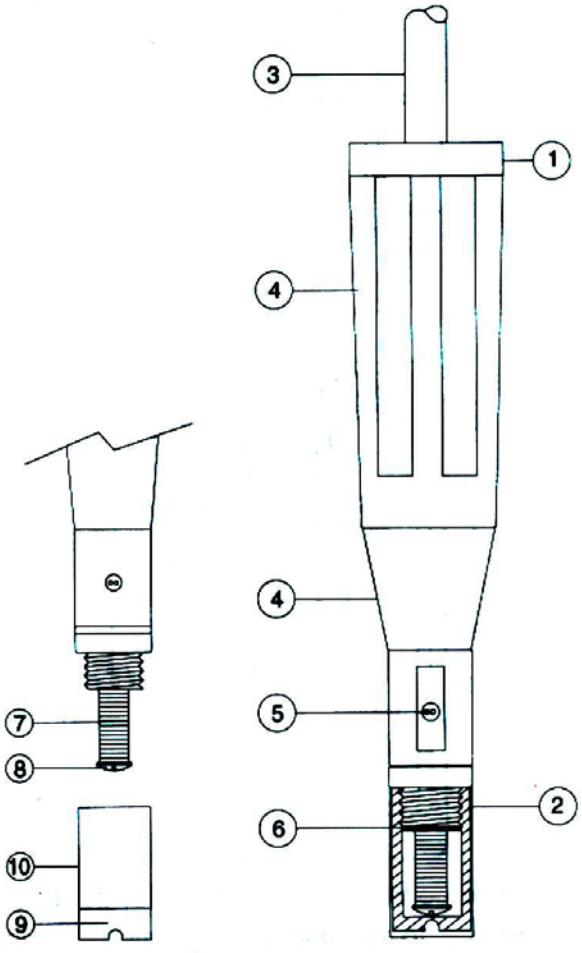


Fig.A.2-3: Scheme of pour plate method.

A.3 O_2-meter.

Measure apparatus coasted of Clark probe that is connected to an electronic meter that measures and displays the O_2 % reading.

In Clark probe for Oxygen detection, a working electrode (platinum) is polarized with a potential -0.8 V (versus silver counter/reference electrode), and current due to the reduction of oxygen is observed. The Clark probe operates in the amperometric mode, with the direct current being measured using simple electronic potentiostat (current-voltage converter). As an analytical signal, current is measured at a constant potential of 0.8-0.9V. For a Clark probe made with a glass pipette diameter of 300 μm, 50 μm platinum anode and 200 μm silver cathode, the current developed is between 3 and 100 pA/μM. Anode and Cathode are immerged in an HCl solution containing in a cap that have at the bottom a gas-permeable Teflon membrane. O_2 in solution pass through Teflon membrane and near cathode it is reduced to OH^-, while on the anode AgCl are deposited.



1. DO probe.
2. Protection cap.
3. Seal cable.
4. Probe body.
5. Thermal sensor.
6. Seal Oring.
7. Silver anode.
8. Platinum anode (sensor).
9. Gas-permeable teflon membrane.
10. Membrane cap.

Fig.A.3-1: Clark probe.

By these reactions derive an electric flux that is proportional of O_2 concentration present in the sample. The current obtains it is transformed in O_2 dissolved concentration and it is expressed as saturation percentage.

For the water solution, the saturation percentage consisted in ratio between real oxygen that is present in sample and theoretic oxygen that is calculated by Benson and Krause equation:

$Ln\ C = -K + 1.57570 * 10^5 /T - 6.642308 * 10^7 /T^2 + 1.243800 * 10/T^3 - 8.621949 + 11/T^4 - S\ (0.01764 - 10.754/T + 2140.7/T^2)$

In which for lnC [cm^3/dm^3] K= - 139.70012, while for lnC [µmol/dm^3] K= -135.90205. T is expressed in K grade.
In the experimental results this analytical methods is used, with approximation of fermented production with water without salinity.

A.4 Total Starch. Assay Procedure.

Starch determination methods are broadly grouped into acid hydrolysis or enzymic procedures. Acid hydrolysis procedures can only be applied to pure starch samples and thus have limited application. Enzymic procedures vary in pre-treatment steps, starch gelatinisation, liquefaction and dextrinisation, hydrolysis of dextrins to glucose, and glucose measurement. AACC method 76-11 specifies starch gelatinisation under aqueous conditions in an autoclave, followed by starch conversion to glucose with amyloglucosidase and glucose measurement.
AACC methods 76-11 underestimates starch content in a range of samples and materials, including high amylase maize starches, and many processed cereal products. Most methods in use today incorporate treatment with thermostable α-amylase either during, or immediately following the starch gelatinisation step. For samples which are difficult to gelatinise (such as high amylase maize starch) solvents such as sodium hydroxide or dimethyl sulphoxide (DMSO) have been employed.

A.4.1 Principle.

The Megazime total starch analysis procedure (AA/AMG) allows the measurement of total starch in most cereal products (natural or processed). In the assay format described, starch hydrolysis proceeds in two phases. In phase I, starch is partially hydrolysed and

totally solubilised. In phase II, the starch dextrins are quantitatively hydrolysed to glucose by amyloglucosidase.

For most samples (e.g. wheat flour), complete solubilisation of starch can be achieved by cooking the sample in the presence of thermostable α-amylase. However, for samples containing high levels of resistant starch (e.g. high amylase maize starch), complete solubilisation and dextrination requires pre-treatment with dimethyl sulphoxide (DMSO) at 100°C.

Sample containing high levels of glucose and maltodextrins are washed with aqueous ethanol before analysis.

Analysis of a single sample can be performed within 70 min.

Twenty samples can be analysed within 2h.

A.4.2 Accuracy.

Standard errors of ± 2% are achieved routinely.

A.5 Lactose and D-Galactose. Assay Procedure.

Lactose, or milk sugar, is a white crystalline disaccharide. It is formed in the mammary glands of all lactating animals and is present in their milk. Lactose yield D-galactose and D-glucose on hydrolysis by lactate (β-galactosidase), an enzyme found in gastric juice. People who lack this enzyme after childhood cannot digest milk and are said to be lactose intolerant. Common symptoms of lactose intolerance include nausea, cramps, gas and diarrhea, which begin about 30 minutes to 2 hours after eating or drinking foods containing lactose.

Enzyme methods for the measurement of lactose are well known and are generally based on the hydrolysis of lactose to D-galactose and D-glucose with β-galactosidase, followed by determination of either D-galactose or D-glucose. In the International Dairy Federation Method (79B:1991) for the measurement of lactose in “dried milk, dried ice-mixes & processed cheese”, details are given for deproteinisation of samples, hydrolysis

of lactose with β-galactosidase and measurement of either released D-galactose or D-glucose. The measurement of lactose as D-galactose liberated is more generally reliable than measurement as D-glucose liberated because preparations generally contain more free D-glucose than free D-galactose.

Enzymic kits for the determination of D-galactose are very slow. This is due to the low rate of natural chemical "mutarotation" between the α- and β- anomeric forms of D-galactose. Only the β- form is recognised by β-galactose dehydrogenase. In incubations containing NAD^+, D-galactose and β-galactose dehydrogenate, there is a very rapid initial increase in absorbance due to the consumption of β-D-galactose, and this is followed by a very slow approach to the end-point. This very slow approach results from the very low rate of chemical "mutarotation" of α-D-galactose into β-D-galactose.

Using technology developed by Megazime (patent pending), a galactose mutarotase has now been incorporated into the assay format to rapidly catalyse this rate-limiting mutarotation step. The result is very rapid analysis times of approx. 5 min at room temperature.

A.5.1 Principle.

In the currently described procedure (a modification of AOAC Official Method 984.15; lactose in milk) lactose in hydrolysed to D-galactose and D-glucose by *Aspergillus niger* β-galactosidase at pH 5.0 (1).

$$\text{(1) Lactose} + H_2O \xrightarrow{\text{(β-galactosidase)}} \text{D-galactose} + \text{D-glucose}$$

Interconversion of the α- and β- anomeric forms of D-galactose is catalysed by galactose mutarotase (GaIM) (2).

$$\text{(2) α-D-galactose} \xleftrightarrow{\text{(GaIM)}} \text{β-D-galactose}$$

The β-D-galactose is oxidised by NAD^+ to D-galactonic acid in the presence of β-galactose dehydrogenate (β-GalDH) at pH 8.6 (3).

$$\text{(3) B-D-galactose} + NAD^+ \xrightarrow{(\beta\text{-GalDH})} \text{D-galactonic acid} + NADH + H^+$$

The amount of NADH formed in this reaction is stoichiometric with the amount of lactose. It is the NADH which is measured by the increase in absorbance at 340 nm.

A.5.2 Specificity, sensitivity, linearity and precision.

The assays are specific for lactose and D-galactose. The smallest differentiating absorbance for assay is 0.010 absorbance units. This corresponds to 1.48 mg of lactose (or 0.74 mg of D-galactose)/L of sample solution at the maximum sample volume of 1.00 mL. The detection limit is 2.96 mg of lactose /L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 1.00mL.
The assay is linear over the range of 4 to 80 µg of D-galactose (or 8 to 160 µg of lactose) per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 1.00 mL, this corresponds to a lactose concentration of approx. 0.74 to 1.48 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100g can be expected.

A.5.3 Interference.

If the conversion of D-galactose has been completed within 10 min at room temperature it can be generally concluded than no interference has occurred. However, this can be further checked by adding D-galactose (approx. 40 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments i.e. by adding lactose or D-galactose to the sample in the initial extraction steps.
To confirm that lactose is completely hydrolysed by β-galactosidase, perform the incubation for the recommended time and for twice the recommended incubation time. The final determined values for lactose should be the same.
Since divalent metal ions inhibit the β-galactose dehydrogenase employed in this assay, EDTA is included in the Tris/HCl buffer.

A.5.4 Safety.

The reagents used in the determination of lactose and D-galactose are not hazardous materials in the sense of the Harzardous Substances Regulations. However, the Tris /HCl buffer concentrate contains sodium azide (0.02 % w/v) as a preservative. The general safety measured that apply to all chemical substances should be adhered to.

A.6 HPLC

High-performance liquid chromatography (or High pressure liquid chromatography, HPLC) (Fig. A.6-1) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. The sample to be analyzed is introduced in small volume to the stream of mobile phase. The analyte's motion through the column is slowed by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile

phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time; the retention time under particular conditions is considered a reasonably unique identifying characteristic of a given analyte. The use of smaller particle size column packing (which creates higher backpressure) increases the linear velocity giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. Common solvents used include any miscible combination of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as trifluoroacetic acid which acts as an ion pairing agent.

The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte. Often a series of tests are performed on the analyte and a number of trial runs may be processed in order to find the HPLC method which gives the best separation of peaks.

A.6.1 Parameters.

A.6.1.1 Internal diameter.

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded onto the column. Larger columns are usually seen in industrial applications, such as the purification of a drug product for later use. Low-ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

- Larger ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity.
- Analytical scale columns (4.6 mm) have been the most common type of columns, though smaller columns are rapidly gaining in popularity. They are used in traditional quantitative analysis of samples and often use a UV-Vis absorbance detector.

- Narrow-bore columns (1-2 mm) are used for applications when more sensitivity is desired either with special UV-vis detectors, fluorescence detection or with other detection methods like liquid chromatography-mass spectrometry.
- Capillary columns (under 0.3 mm) are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

A.6.1.2 Particle size

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5 μm beads being the most common. Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared (Xiang Y. et al., 2006; Horváth Cs. Et al., 1967).
This means that changing to particles that are half as big, keeping the size of the column the same, will double the performance, but increase the required pressure by a factor of four. Larger particles are used in preparative HPLC (column diameters 5 cm up to >30 cm) and for non-HPLC applications such as solid-phase extraction.

A.6.1.3 Pore size

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes. For example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside.

A.6.1.4 Pump pressure

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 40 MPa (6000 lbf/in^2), or about 400 atmospheres. Modern HPLC systems have been improved to work at much higher pressures, and therefore are able to use much smaller particle sizes in the columns (<2 μm). These "Ultra High Performance Liquid Chromatography" systems or RSLC/UHPLCs can work at up to 100 MPa (15,000 lbf/in²), or about 1000 atmospheres. The term "UPLC", though sometimes used is a trademark of Waters Corporation and not the name for the technique in general.

A.6.2 Instrumentation

Solvents must be degassed to eliminate formation of bubbles. The pumps provide a steady high pressure with no pulsating, and can be programmed to vary the composition of the solvent during the course of the separation. Detectors rely on a change in refractive index, UV-VIS absorption, or fluorescence after excitation with a suitable wavelength.

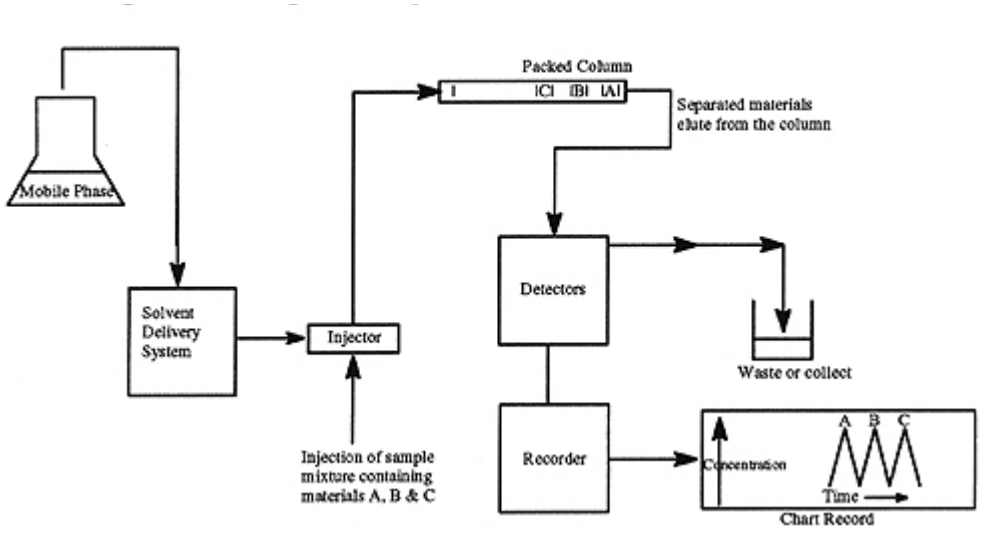


Fig.A.6-1: Schematic of an HPLC instrument.

A.7 Gas chromatograph.

Gas-liquid chromatography (GLC), or simply gas chromatography (GC), is a common type of chromatography used in analytic chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can also be determined). In some situations, GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture.

In gas chromatography, the *moving phase* (or "mobile phase") is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The *stationary phase* is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column (an homage to the fractionating column used in distillation). The instrument used to perform gas chromatography is called a *gas chromatograph* (or "aerograph", "gas separator").

The gaseous compounds being analyzed interact with the walls of the column, which is coated with different stationary phases. This causes each compound to elute at a different time, known as the *retention time* of the compound. The comparison of retention times is what gives GC its analytical usefulness.

Gas chromatography is in principle similar to column chromatography (as well as other forms of chromatography, such as HPLC, TLC), but has several notable differences. Firstly, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas moving phase, whereas in column chromatography the stationary phase is a solid and the moving phase is a liquid. (Hence the full name of the procedure is "Gas-liquid chromatography", referring to the mobile and stationary phases, respectively.) Secondly, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography (typically) has no such temperature control. Thirdly, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas (Pavia et al., 2006).

Gas chromatography is also similar to fractional distillation, since both processes separate the components of a mixture primarily based on boiling point (or vapor pressure) differences. However, fractional distillation is typically used to separate components of a mixture on a large scale, whereas GC can be used on a much smaller scale (i.e. microscale).

A.7.1 GC analysis

A gas chromatograph (fig. A.7-1) is a chemical analysis instrument for separating chemicals in a complex sample. A gas chromatograph uses a flow-through narrow tube known as the *column*, through which different chemical constituents of a sample pass in a gas stream (carrier gas, *mobile phase*) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the *stationary phase*. As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (*retention time*). Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, and the temperature.

In a GC analysis, a known volume of gaseous or liquid analyte is injected into the "entrance" (head) of the column, usually using a microsyringe (or, solid phase microextraction fibers, or a gas source switching system). As the carrier gas sweeps the analyte molecules through the column, this motion is inhibited by the adsorption of the analyte molecules either onto the column walls or onto packing materials in the column. The rate at which the molecules progress along the column depends on the strength of adsorption, which in turn depends on the type of molecule and on the stationary phase materials. Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column and reach the end of the column at different times (retention time). A detector is used to monitor the outlet stream from the column; thus, the time at which each component reaches the outlet and the amount of that component can be determined. Generally,

substances are identified (qualitatively) by the order in which they emerge (elute) from the column and by the retention time of the analyte in the column.

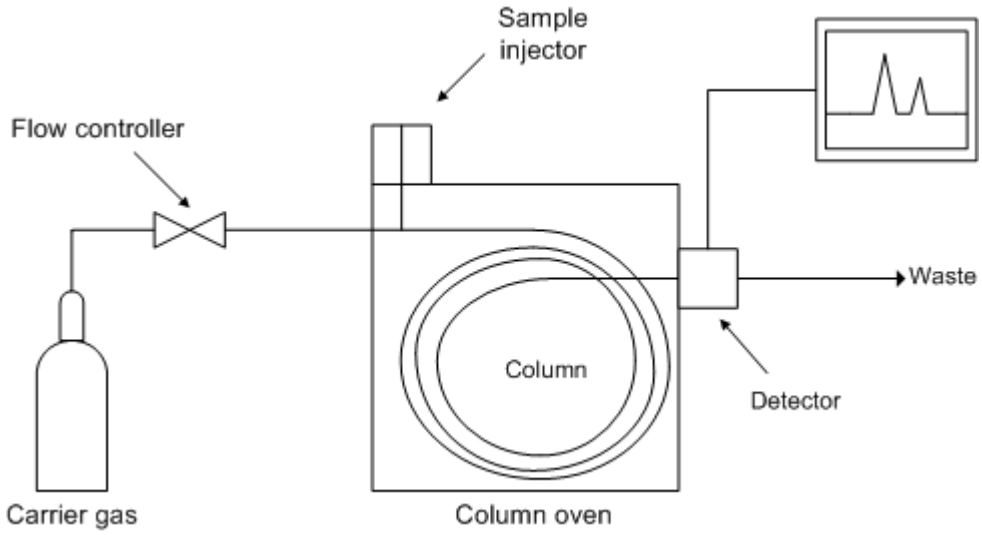


Fig.A.7-1: Diagram of a gas chromatograph.

A.8 Real time PCR.

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction, is a laboratory technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle.

Real time quantitative PCR uses fluorophores in order to detect levels of gene expression.

Cells in all organisms regulate gene expression and turnover of gene transcripts (messenger RNA, abbreviated to mRNA), and the number of copies of an mRNA transcript of a gene in a cell or tissue is determined by the rates of its expression and degradation.

Northern blotting is often used to estimate the expression level of a gene by visualizing the abundance of its mRNA transcript in a sample. In this method, purified RNA is separated by agarose gel electrophoresis, transferred to a solid matrix (such as a nylon membrane), and probed with a specific DNA or RNA probe that is complementary to the gene of interest. Although this technique is still used to assess gene expression, it requires relatively large amounts of RNA and provides only qualitative or semiquantitative information of mRNA levels.

In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The polymerase chain reaction is a common method for amplifying DNA; for mRNA-based PCR the RNA sample is first reverse transcribed to cDNA with reverse transcriptase.

Development of PCR technologies based on reverse transcription and *fluorophores* permits measurement of DNA amplification during PCR in real time, i.e., the amplified product is measured at each PCR cycle. The data thus generated can be analyzed by computer software to calculate *relative gene expression* in several samples, or *mRNA copy number*. Real-time PCR can also be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples.

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