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Fermentation processes for bioinsecticide production. An overview

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Abstract

Bacillus thuringiensis (*Bt*) accounts for more than 90% of the bioinsecticides used today. It is effective against larvae of various insects of lepidoptera, coleoptera and diptera orders, due to the production of δ-endotoxins (Cry) during sporulation phase. For *Bt* production: batch, fed-batch and continuos cultures have been studied. Among them, batch culture renders the highest Cry:spore ratio, which increases specific insecticidal activity.

Complex nutrient sources and oxygen supply play important roles in the sporulation and Cry production. Results obtained in batch cultures by our group suggests an interaction between carbon:nitrogen ratio and the initial concentration of total solids which affect Cry:spore ratio. Additionaly high specific growth rate lowers Cry:spore ratio.

Understanding the correlation among *cry* gene expression, *Bt* physiology and fermentation parameters could allow the development of fed-batch culture with Cry:spore ratio comparable to that of batch culture.

Paecilomyces fumosoroseus is a powerful biocontrol agent against several pest insects, including the white flies *Bemisia tabaci* and *B. argentifolii*. Conidia of this fungus are currently produced in Mexico by solid state fermentation. Several groups have focused on submerged culture in order to increase productivity and improve process control to reduce costs. Some patents have been issued covering submerged production of blastospores and mycelium, which are less resistant to adverse environmental conditions than aerial conidia. Our group first reported production of conidia in submerged culture. *Pfr* production is dependent on several operating conditions, as media composition. Optimisation of these conditions will let to reduce production costs without affecting resistance and virulence of the fungus.

Among nematodes, *Steinernema* and *Heterorhabditis* genus have been the most investigated for biological control. These nematodes show symbiotic relationships with certain bacteria. Under some conditions not totally known J3 stages (non-resistant juvenile) transform into the resistant infective juvenile (IJ) stages that may survive outside the host and have the capacity to find and invade a prey. Once inside the insect the pathogenicity depends on the presence of the symbiotic bacteria. For IJ mass production, *in vivo* and *in vitro* nematode cultures have been studied, among the second ones, the submerged monoxenic culture seems to be the most suitable for the large-scale production, due to its high productivity and relatively easy process control. Although industrial production of IJ has been achieved for some nematode species, there is still a lack of basic knowledge for the proper

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bioreactor design. This is the case of knowledge concerning the propagation kinetics involved, the hydrodynamic effects on viability and mating process, and the role of oxygen transfer rate for the final nematode concentration achieved.

State of the art, trends and relevant results of our laboratory are discussed.

1 Introduction

Since the late 1940's insect control has been based on synthetic chemical insecticides. However, because of its undesirable side effects there is growing interest among farmers, gardeners, government and ecological groups to use integrated pest management (IPM) approaches for reduction of chemical pesticide use. Biological control is an alternative and biotechnology provides the tools for mass production of microbial control agents.

Currently, insect eradication programs, which rely heavily on the use of pesticides, constitute a multi-billion dollar global annual market. Microbial agents, mainly *Bacillus thuringiensis*, have sequestered a minor fraction of this market. IPM is becoming widespread in current agricultural practices, due to the ever-increasing resistance to chemical insecticides shown by insect populations. IPM implies the combined use of different strategies, among them is biological control. However, growth in market share will continue as dictated by commercial, legislative and ecological factors and other suitable biological pest control candidates will set off. These include bacteria, fugi, nematodes, protozoans and various viruses.

Some of the constrains for wide use of microbial agents for biological control are their production cost and the lack of knowledge on the effect of the process conditions for their mass production on product virulence and shelf life. The aim of this review is to discuss current knowledge of some processes variables relevant for mass production of *Bacillus thuringiensis*, the entomopathogenic fungi *Paecilomyces fumosoroseus* and the nematode *Steinernema feltiae*.

2 *Bacillus thuringiensis*

Bt is a pathogen for early larval stages of lepidoptera, diptera and coleoptera as well as some adult coleoptera, although there are some varieties with reported activities against nematodes [1], lice [2] and mites [3]. The bacteria produce a parasporal crystal during sporulation and the larvae ingest both the spore and the parasporal crystal. The crystal is dissolved by the strong alkaline medium of the insect intestine (pH=10) and the proteins are partially processed by digestive proteases to protease-resistant and highly toxic polypeptides known as δ-endotoxins [4]. Endotoxins bind to specific receptors of epithelial cells and generate ion channels that conduce to cell lysis and spore germination. Insects die either by starvation or septicaemia [5].

Some lepidoptera are insensitive to isolated crystals. The reason for this resistance deals with an intestinal pH inadequate for the dissolution of the crystal or a deficient processing of the protoxin by digestive proteases, which renders an inactive toxin. Therefore, solubility of the crystals is a clue for the biological activity of *Bt* [6].

The *Bt* parasporal crystal can account for up to 25% of the sporulated cell dry weight. To produce such a high crystal concentration the cells have to synthesise about 1 or 2 million δ-endotoxin molecules during the stationary growth phase. *Bt* posses several mechanisms to achieve this remarkable Cry protein over-expression and accumulation [7]. The *cry* genes are usually located in high-weight plasmids of more than 200 mDa and frequently there exists multiple copies.

It has been demonstrated that the range and intensity of toxicity shown by a given *Bt* strain depends on the aminoacid source used to grow the microorganism [8]. Our research group has suggested that it could be due to a phenomenon of differential regulation of the *cry* genes, appearing when different substrates are used. This would provoke a variable proportion of crystal composing-proteins, which would in its turn modify the solubility and toxicity of the crystals [9]. Cheng and Aronson suggest that the regulation of expression of the individual *cry* genes is probably important for determining the overall toxicity profile of an isolate [10].

Our research is focused on the effect of process conditions on Cry:spore ratio. Production alterna-

tives and our recent results will be discussed.

2.1 Factors affecting Cry production

Commercial production of *Bt* is performed using culture media based on complex nutrient sources. Spore counts, toxic potency and toxicity ranges of the product have been reported to depend on the culture medium [8, 11, 12, 13]. Insecticidal activity of the crystal produced by the same *Bt* strain can be affected by culture medium and operating conditions. Besides, not always a high cell growth ensures an elevated Cry protein production or an increased insecticidal activity. Dulmage [11] reported a great variability in *Bt* cultures toxicity when using two different varieties of the bacterium and two different media, one based on tryptone and corn meal and the other based on bactopeptone and cottonseed meal. The toxicity of the same strain changed from 206 to 680 international units per mL.

2.1.1 Carbon source

Glucose is by far the most appropriate carbon source either for growth or for sporulation. [12, 14, 15]. Without readily assimilable carbohydrates, defective sporulation occurs in most cases [16]. There are experimental results indicating that the change in the carbon source affects the biological activity and the morphology of the crystals obtained from the same strains, possibly due to alteration on the rate of synthesis of the different *Cry* proteins [13]. A deficiency of glucose in the medium without an alternative carbon source may trigger sporulation. Alternatively, glucose present at the end of fermentation can provoke a disynchronization of sporulation [15]. Rajalakshmi and Shetna [17], reported that in batch process, sporulation is totally inhibited by the presence of cistine along with glucose and other nutrients. Our data [18] confirmed that sporulation can be triggered alternatively by glucose or aminoacid deficiencies.

In a recent study Yang and Wang [19], investigated the specific phase for optimisation of the toxin expression Cryl y CryII from genetically engineered *Bt*, varying nutrients and environmental parameters. Fundamentally they discovered that the existence of nitrogen sources in medium during the toxin-expression phase is detrimental to toxin-protein expression, whereas a high carbon level (40 $g \cdot L^{-1}$) encouraged protein expression. It indicates that the depletion of the nitrogen source is the trigger for *Bt* to start the sporulation and toxin expression.

2.1.2 Nitrogen source

To obtain a good sporulation and parasporal crystal formation in *Bt*, free aminoacids have to be supplied in the medium [16, 20]. Its absence provokes a delayed sporulation and a low yield of protoxin, with the consequent low biological activity [21, 22]. The role of these free aminoacids is not clear. Probably they fulfil the role of rapid sources of nitrogen in early stages of fermentation, besides the fact that they can be used as carbon sources [23, 24, 25]. Amino acids used for crystal synthesis are not taken directly from the medium, but come from the turnover of structural proteins of the sporangium [26].

Sakharova et al. [25], reported that *Bt* uses as a carbon source, first amino acids, then glucose and later amino acids again and observed a diauxic growth pattern. Anderson [27] reported a diauxic growth, too. Our research team has not found evidence of diauxic growth or sequential assimilation of glucose and amino acids in *Bt kurstaki* HD-1 and HD-73. On the contrary, a simultaneous consumption of glucose and amino acids have been observed [28].

The fact that protein-rich media can inhibit sporulation has been stated. Pearson and Ward [29] have even recommended protein-rich media for inoculum development, where sporulation is undesirable. Egorov et al. indicated that both protease and spore production in several strains seem to be regulated by nitrogen [20, 23].

2.1.3 Carbon:Nitrogen ratio

Anderson [27] suggested a relationship between Carbon and nitrogen ratio (C:N) and *Cry* production. He found that in a media with low C:N ratio, the biomass yield based on glucose ($Y_{X/S}$) was higher than 1 suggesting an extent use of amino acids as carbon and energy sources. At high C:N, glucose did not deplete at the end of the fermentation and biomass yield decreased. Finally, in balanced media (C:N of 7.5:1) $Y_{X/S}$ and

glucose consumption achieved maximum values. Additionally he found that, when using oxygen transfer coefficients (k_La) close to 120 h^{-1} the above described influence of C:N was negligible and suggested that the relative importance of balancing the medium to obtain high Cry production depended on the existence or not of a good oxygenation. Similarly, Dulmage et al. [8] insisted in the need of balancing carbon and nitrogen sources to avoid pH lower than 5.8 in the middle fermentation or higher than 8 at its end, which can alternatively inhibit growth or dissolve crystals. We found that a C:N of 7:1 in the medium improved the Cry production for any given initial concentration of total solids (C_{ST}) value. Maximum spore production was affected by C:N values, depending of the C_{ST} [18] and oxygen availability during the different culture phases. The mechanisms through which these effects take place remain to be elucidated. Competition between different *cry* genes, and other sporulation genes for the transcription and translation elements may be part of the explanation. Also the appropriate onset of the metabolic fluxes in the functional routes during sporulation, the availability of energy, and the adequate balance between reduced and oxidised coenzymes might be involved.

2.1.4 Specific growth rate

Maximum specific growth rate (μ_{max}) reported for several *Bt* strains vary from 0.29 to 1.9 h^{-1}, with the most common value close to 0.8 h^{-1} [30]. Our research group have found that a high specific growth rate (μ) of vegetative cells is counter-productive for Cry production [31], possibly due to accentuated plasmid loss. Alternatively, it might be that under high μ the cell reaches a physiological condition in which the metabolic effort is directed to reproduction and not to accumulate reserve materials. Specifically, poly-β-hydroxybutirate (PHB) apparently acts as the source of energy for sporulation and Cry synthesis. With this scheme, high μ values would lead to cultures with high spore count but low toxicity.

2.1.5 Oxygen

High aeration rates are essential for spore and toxin formation [21, 27, 29, 32]. Rowe and Margaritis [33] mentioned that during transition and sporulation phases the oxygen demand is only 30% compared to the exponential phase. However, Anderson [27] found that the oxygen demand in the vegetative phase was lower than in the transition and sporulation phases. He also reported that Cry production and biomass increased with k_La. Arcas [34] stated a proportionality between sporulation and oxygen transfer coefficient (k_La). Razo-Flores et al. [35], in our laboratory, found a lineal correlation between *Bt* spore production and k_La, and scaled-up the process from 14 L to 1000 L, using constant $k_La \cdot p$ (where p is the fermentor total pressure) as the scale-up criterion. In the biggest fermentor μ decreased, but spore productivity and spore yield remained constant.

Arcas et al. [14] found a correlation among initial glucose concentration, aeration and spore yield. If initial glucose was elevated to 56 $g \cdot L^{-1}$ and the medium was vigorously shaken to maintain a dissolved oxygen (DO) over 20% of saturation with air, spore counts of 7.36 x 10^9 spores$\cdot mL^{-1}$ for batch and up to 1.2 x 10^{10} spores$\cdot mL^{-1}$ for fed-batch could be obtained [36].

It is probable that the high oxygen transfer prevents the excessive accumulation of organic acids produced by glucose catabolism, increasing the fluxes of the first part of the Krebs cycle, and therefore, pH does not decrease drastically. Nevertheless, the correlation can be more complex. Results obtained in our laboratory suggested a relationship between oxygen availability, storage of PHB and Cry protein production [28]. Since 1982, Wakisaka et al. [37] reported a correlation between PHB granules apparition and δ-endotoxin formation in *Bt*. They found that at low potassium concentrations of 11 $\mu g \cdot mL^{-1}$ no δ-endotoxin was formed but large granules of PHB accumulate. At medium concentrations of 60 to 100 $\mu g \cdot mL^{-1}$ either PHB granules or δ-endotoxin could be found in sporulated cells, but total biomass decreased. Finally, at high potassium values over 100 $\mu g \cdot mL^{-1}$ PHB was totally consumed and δ-endotoxin production was normal.

2.1.6 pH

Optimum pH for *Bt* growth is found between 6.8

and 7.2. In the early stages and during the vegetative growth, the predomination of glycolysis and the accumulation of lactate, piruvate and acetate cause a descent in pH. Growth inhibition, bacilli malformations and defective sporulation occur if pH fells below 5.8. If pH rises to 9 crystal can be dissolved. During transition, fatty acids are consumed and pH goes up. Through sporulation amino acids are metabolised, and amino compounds are released to the medium, raising the pH even more. At the end of fermentation, pH can reach 8.3 or higher. In 1982, Smith found that final pH in the range of 5.3 to 8.1 does not affect the level of Cry produced [12]. Additionally, it has been suggested that an elevated aeration enhances the acid metabolite consumption and avoids the excessive pH descent [38].

2.1.7 Temperature

For optimum Cry production, *Bt* must be cultured between 28°C and 32°C. High temperatures increase plasmid losses, favouring the selection of acrystalliferous and asporogenic mutants [33, 34].

2.2 *Bt* production alternatives

In 1999, the United States Environmental Protection Agency (EPA) has registered 167 bioinsecticides formulated with *Bt* [39]. Nowadays *Bt* is produced at industrial level by submerged fermentation using complex media [4]. The efficiency of the process has been measured as spore production, although no relationship between spore count and Cry has been demonstrated. Therefore, at the laboratory and pilot plant scales batch, fed-batch and continuous culture have been studied. A brief summary of each option is presented.

2.2.1 Batch fermentation

According to Dulmage et al. [8] the industrial fermentation method of choice is the batch culture, with the following advantages: a) plasmid losses are minimised; b) appearance of acrystalliferous and asporo-genic mutants, as well as cell lysis due to phages are reduced [16]; c) it is relatively simple to achieve sporulation synchronisation because the lack of nutrients triggers sporulation; d) product biological activity is higher compared to that obtained with other culture systems.

Industrial process conditions were reviewed by Farrera and de la Torre [4]. In the typical process the total nutrient concentrations have gone up to 10% w/v. The fermentation time ranges between 24 h and 36 h and in certain cases may extend to 72 h. Spore concentrations of 10^8 and 10^9 spores per mL of broth have been obtained. Data on Cry concentration are not available.

Downstream processing differs depending on the producer and product. In the cases of aqueous concentrates, the fermented broth is concentrated and washed through centrifugation, and then the product is formulated, standardised, dosaged and packed in plastic or metal vessels. Filtering with a filter aid is also an economical alternative [40]. If the product is an oil-based concentrate, the washed and centrifuged cream is mixed with an oil-based suspension during formulation. If the presentation is a dry wettable powder the broth is concentrated and washed through centrifugation, preformulated with preservatives, humidifiers or dispersants and spray-dried. The powder is used for formulation and packed. Diluents, UV protectors, insect attractors, adhesives, flow enhancers and other additives are used for the final formulation.

Batch fermentation researches have been conducted with the goal of achieving the maximum spore concentration, rather than toxin-protein concentration [19]. Frequently this information is variety-related and seems contradictory [18]. Arcas et al. mentioned that the degree of sporulation observed in the batch fermentation was directly associated with endotoxin production [36]. They observed an increase in spore counts from 1.08×10^9 to 7.36×10^9 spores mL^{-1}, and toxin level from 1.05 $g \cdot L^{-1}$ to 6.85 $g \cdot L^{-1}$ when the glucose concentration was increased from 8 $g \cdot L^{-1}$ to 56 $g \cdot L^{-1}$, with a correspondence increase in the rest of medium composition. However our research group found that the best conditions for spore production were different from those for Cry production [18], supporting other reports that high final spore counts are not proportional to high toxicities [41]. We also encountered that level of C_{TS} up to 150 $g \cdot L^{-1}$ can be used to grow *Bt kurstaki* HD-73 with a greater productivity of Cry in batch culture, counts of 15.5×10^9 spore$\cdot mL^{-1}$ and Cry production of 2.66 $g \cdot L^{-1}$ [18].

Traditionally, investigations of *Bt* in batch culture are carried out in both stirred tank and pneumatically agitated reactors [5, 42]. Although the cell densities and Cry production reached in those systems are similar. Our research group produce *Bt* in a 10 m^3 jet loop fermentor that was designed for processes that require high oxygen transfer rate [43].

2.2.2 Fed-batch fermentations

This culture is a batch in which μ is controlled by feeding fresh concentrated media to a vessel, which slowly increases its working volume. Constant, variable or intermittent feedings can be used. The advantages of this method involve the minimisation of substrate or catabolic inhibition effects and the achievement of high cell concentrations. An increase in cell concentration could imply an increase in Cry concentration. Our team has performed fed-batch experiments with *Bt kurstaki* HD-73, in which concentrations of 2.7 x 10^{10} spores·mL^{-1} have been achieved in 23.5 h. In some of our fed-batch experiments, glucose accumulation and the biomass yield based on substrate indicated that the bacterium used amino acids as another carbon source [44].

Fed-batch *Bt* fermentation do not seem to favour Cry protein production. Kang et al. [45], working with *Bt kurstaki* HD-1 under an intermittent feed method found that high glucose concentration rendered high biomass growth but low sporulation and concluded that in fed-batch, fast growth enhanced sporulation. Avignone-Rossa and Mignone [41] compared simple batch and fed-batch fermentations of *Bt israelensis* and found that in spite of similar biomass and spore concentrations, the toxicity of the fed-batch product was much lower. They concluded that fed-batch produces high spore counts but diminish notably the associated toxicity and assumed that the toxic component synthesis was affected if an exponential growth was not achieved.

Jong et al. developed a glucose adaptive control for substrate addition in fed-batch culture of *Bt darmstadiensis* HD-199 for soluble exotoxin production [42]. They reached 1.5 x 10^{10} spores mL^{-1} and 11.7 exotoxin $g{\cdot}L^{-1}$ in fed-batch experiments. Protein production was seven times higher that in batch culture.

To find the needed conditions for obtaining a high Cry:spore ratio in fed-batch cultures is a difficult challenge. The following questions have not been answered yet: a) How defeat composition should be balanced to get a maximum Cry production? b) What is the desired μ to obtain a maximum Cry production?

2.2.3 Continuous culture

With continuous culture it is possible to maintain a microbial population in a steady condition and manipulate μ and the physiological state of the microorganism. On the contrary, prolonged culture periods of *Bt* favour the most energy-efficient mutants that frequently are not the overproducers, as well as the appearance of acrystalliferous mutants [46]. Blokhina and co-workers [47] found that in continuous culture *Bt* dissociates into R ("Rough") and S ("Smooth") variants, with significant differences in their sporulation, toxin synthesis and phage resistance capacities. Some authors [48, 49] have stated a high phage lysis susceptibility of *Bt* strains. Khovrychev et al. [50], analysed the phage title during continuous culture of *Bt galleriae*, and found it to be constant, in the range of 10^6 to 10^7 per mL, which do not cause an extent lysis of the culture.

Several studies have been performed on *Bt* continuous culture in staged systems with at least one stage dedicated to vegetative growth and another to sporulation. Khovrychev et al. [50] used a two-stage culture of *Bt galleriae*, with decreasing the dilution rate (D) to D_1/D_2 of 4 with limitation of glucose. They kept the culture for 15 d without asporogenic mutant selection, but only 20% to 30% of the bacilli sporulated. Since the total residence time in both stages was of only 20 h and the microorganism required 27 h to complete its cycle, many cells could be eluted out from the system before being able to sporulate. Later they used a three-stage system with dilutions D_1:D_2:D_3 of 8:2:1, which was kept up to 11 d, but not in a steady state. With this system they obtained 30% sporulation in the second stage and 80% in the third. If dilution at the third stage was reduced to obtain a total residence time of 60 h, sporulation ratio decreased due to spore germination. As an interesting fact, they mentioned the finding of crystal-containing cells without spore. The R and S variant ratio and the

phage title remained constant while increasing the number of stages [51].

Our group performed continuous cultures of *Bt kurstaki* HD-73 in a single-stage system with different dilution rates [30]. Steady states were achieved in all cases and three different metabolic states were observed, according to biomass yield based on glucose and microscopic examination. Between a D of 0.18 h^{-1} and 0.31 h^{-1} vegetative cell, sporulating bacteria and free spores coexisted while both glucose and aminoacids from the medium were consumed. With a D among 0.42 h^{-1} and 0.47 h^{-1} glucose was the main carbon source and only vegetative cells were observed. At a D of 0.5 h^{-1} the biomass yield based on substrate decreased, indicating a change in metabolism. When varying the glucose concentration in the feed from 20 to 30 $g \cdot L^{-1}$ the D value interval in which only vegetative cells existed and mainly glucose was consumed broadened to 0.24 h^{-1} and 0.45 h^{-1} [30].

In 1997, Shachidanandham et al. [52] mentioned that in continuous cultures, a major fraction of *Bt* population entered sporulation, as reflected by the elevated spore count at low D, which decreased at higher D values. Continuous cultures at high D nearly represents the exponential phase in batch cultures, which supports high volumetric biomass output. Since a number of sporulation-associated functions and genes coding for crystal proteins in *Bt* are expressed only during the post-exponential phase, single stage continuous cultures, operated a high D, do not support the formation of spores and crystal protein [53].

2.3 Conclusions

The insecticidal *Bt* preparations have to include viable spores and sufficiently toxic crystals, to ensure the biological activity against the selected target insects. To achieve this, from the bioprocess point of view, it is necessary to understand the correlation among *cry* gene expression, *Bt* physiology and fermentation parameters. This could allow the development fed-batch culture with Cry:spore ratio comparable or higher to that of batch culture.

In such a case it would be possible to design and optimise the processes to desired objectives, like the production of a parasporal crystal with especial characteristics, like a constant proportion of cry proteins or an specific solubility. Yang and Wang suggested three critical areas: a) process study with genetically engineered multiple-toxin producers; b) process optimization that specifically improves toxin-protein yield; and identification of factors that specifically influence the sporulation and toxin-expression phase [19].

3 *Paecilomyces fumosoroseus*

Several entomopathogenic fungi-based pesticides are now available on the market. Fungi species produced in Latin-America include *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus* (*Pfr*), *P. lilacinus*, *Entomophthora virulenta, and Verticillium lecanii*, (AGROBIONSA, Culiacán, Sinaloa, México; INISAV, La Havana, Cuba; LAVERLAM S.A., Cali, Cali, Colombia; PROBIOAGRO, Acarigua, Portuguesa, Venezuela). Fungi posses certain characteristics which makes them especially useful for biological control. One unique characteristic is their ability to penetrate the host insect's epicuticle, making them the only effective microbial insecticide for piercing and sucking insect pests [54]. Fungi also have certain degree of specificity, which allows decreasing an insect plague population without damaging other species, including beneficial insects. Furthermore, many species of predatory insects seem refractory to fungal infections when challenged in the laboratory [55]. Another advantage is their capacity to produce several toxins and enzymes to fight their host's defences [56]. These fungal metabolites are also interesting for genetic improvement of the same fungi and other entomopathogens.

Interest in commercial development of *Paecilomyces fumosoroseus* (Wize) Brown & Smith (Deuteromycotina: Hyphomycetes) began on the late 1980's when sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) and specially silverleaf whitefly *Bemisia argentifolii* Bellows & Perring (Homoptera: Aleyrodidae) rapidly spread and became a serious pest for both field and greenhouses crops. The damage currently inflicted world-wide is estimated at hundreds of millions of dollars annually [57]. Economic losses attributed to *Bemisia argentifolii* in the United

States (US) alone were estimated at USD $1 billion in 1992 [58]. Another plague susceptible to *Pfr*, the Russian wheat aphid *Diuraphis noxia* Kurdjumov (Homoptera: Aphididae), has caused a combined direct and indirect economic impact in excess of USD $1 billion since the mid 1980's, only in the US [58].

Pfr has been repeatedly isolated from *Bemisia* spp. and other important pests, is one of the most ubiquitous and important pathogens of *Bemisia* spp. world-wide [59]. Its capacity to control specific insect pest populations both in greenhouses and field is proved [60], moreover, *Pfr* is well adapted to several different climates [61]. Studies have demonstrated that *Pfr* can be used in combination with predators and parasitoids of insect plagues, an advantage over wider host spectrum fungi as *M. anisopliae* or *B. bassiana* [55, 62, 63, 64]. In view of its potential, a patent was issued in 1990 [65], claiming for *Pfr* strain Apopka (ATCC 20874), compositions including it, and process for controlling 12 insect plagues and their relatives. In Sinaloa and other Mexican states, a product formulated with a different strain of *Pfr* (Pae-Sin®, AGROBIONSA), has demonstrated its effectiveness for controlling *Bemisia* spp., surpassing *M. anisopliae*, *B. bassiana* and *Verticillium lecanii* on the field (AGROBIONSA, unpublished data).

One of the most concerning requisites for production and application of entomopathogens is safety. Although there are no reports of *Pfr* infecting humans, there are two isolated cases in animals since 1962 (Josep Guarro, personal communication), and other species of the genus *Paecilomyces* may infect immunocompromised human patients [66]. On the other hand, *Pfr* has been applied to crops at greenhouses in the U.S.A., and fields in Mexico for more than five years without incidents.

Fungi may be obtained in different morphological forms (mycelium, conidia and blastospores) depending on the production process. Here we review the actual knowledge and our recent findings on the relevant factors for mass-production of those different morphological forms. Each one has advantages and disadvantages for biological control and down-stream processing. Since high virulence and stability are desirable for an effective microbial insecticide, and low production costs are determinant for commercial success, morphology plays a key role on the mycoinsecticide production.

3.1 Mycelium

Pfr can grow as mycelium both in surface and submerged culture. Carbon and nitrogen source and C:N, as well as other nutrients, pH, humidity, oxygen, temperature and the presence of light affects mycelium growth. *Pfr* grows well on complex media like potato-dextrose-agar and Sabouraud-agar, and on some defined media. Its maximum radial growth rate on a defined medium-agar varies between 3.30 ± 0.06 $mm \cdot d^{-1}$ and 5.15 ± 0.08 $mm \cdot d^{-1}$ at optimal temperature, which varies for different strains from 20°C to 30°C [61].

In submerged culture *Pfr* may grow as filaments, or may aggregate into entangled filaments or pellets. Pellets of this fungus are commonly formed at the beginning of the fermentation by the aggregation of the aerial conidia used as inoculum, so when conidia germinate, the mycelium grows radially from the centre. Pellet size and number can be reduced by agitation [67]. Pellet formation may be avoided by the addition of a detergent, as Tween 80®, or using blastospores or mycelium instead of conidia; and may be promoted by the presence of droplets of an hydrophobic phase like antifoam (Fernández-Sumano, unpublished data). If antifoam is not added, filamentous mycelium may be kept trapped in the foam and all the biomass in suspension removed in short time. Morphology is also strongly affected by medium composition, pH, kind of propagule used to inoculate, and initial concentration of propagules (Fernández-Sumano, unpublished data). Understanding the relations between process variables and morphology of this and other fungi is very relevant to the mass production process design for three reasons: a) there might be diffusional limitations of one or more substrates (specially oxygen) inside the aggregate; b) morphology modifies broth rheology [68], thereby affecting production costs by changing mixing energy input requirements, and c) pellet morphology can greatly simplify biomass recovery.

Mycelium may be used as a bioinsecticide product. Eyal et al. [67] developed a process to

produce *Pfr* in submerged culture. They used a 20-L fermenter (16-L V_L) and obtained 30 $g \cdot L^{-1}$ dry weight of biomass, in which at least 80% of the biomass was in the form of filamentous mycelium. To avoid hyphae aggregation, agitation was between 400 rpm to 600 rpm (impeller design is not reported) and aeration between 0.8 vvm and 1 vvm. Fermentation was completed after 96 h to 100 h, the biomass was harvested by centrifugation and mixed with a carrier (alginate), formed into prill and dried to 6-10% moisture in a fluidised bed below 30°C. Each prill (less than 1 mm diameter) can produce as much as 10^7-10^8 conidia after addition of water. Prill can be stored 12 months at 25°C and retain 95% viability. One disadvantage is that mycelium in the prill must produce conidia before insects can be infected and killed (if conidia ever reaches them), and this could take several days. To date we do not know of a commercially available product based on this process.

3.2 Aerial conidia

Aerial conidia are the natural resistance and spreading form of the fungus. These are produced on the surface of the dead insects or any other suitable media. Aerial conidia of several fungi are the desired insecticide product because of their greater virulence and resistance to adverse environmental conditions (UV exposure and dessication) [69, 70, 71].

Conidia of *Pfr* are differentiated asexual spores, oblong in shape, very hydrophobic and rugose. The surface of *Pfr* conidia consists of a bare protein-rich hydrophobic rodlet layer that interacts with insect epicuticle for attachment [72]. It is reasonable to expect that the warty brittle outer layer of aerial conidia confers the extra protection that makes them more resistant to adverse environmental conditions [69]. Altre et al. [72] reported a direct correlation between conidial length (2.9 μm to 5.3 μm) of different strains cultivated on the same medium and infectivity index. We have found that length of conidia also varies when *Pfr* is cultivated in different media, and larger conidia grown in richer media stays viable longer stored on filter paper at 4°C.

Methods for producing aerial conidia are well known [73]. In solid-state fermentation, the fungi are inoculated to a solid or semisolid media in trays, bags, or packed columns and allowed to grow and conidiate, process that takes about 2 weeks. Two-stage fermentation involves growing fungi in liquid media followed by inoculation of a solid media or an inert carrier on which the conidia are produced.

When protected from light, mycelium of the strain PFRD (ARSEF 3302) growing on Sabouraud-Agar plates completely fills the Petri's dish and produce few conidia, but when exposed to light, it grows less, changes its colonial morphology, shows phototropism and conidiates abundantly. Growth and conidiation of other strains are not affected when growing in the dark [74].

Solid-state fermentation allows a fungus to produce hardy, healthy conidia [75], but is very labour intensive, and control of critical parameters as temperature and humidity is difficult. Mycotech Corp. [59] found that conidia yields of *B. bassiana* were approximately fourfold greater than those of *Paecilomyces* spp. under pilot-plant scale production conditions. However, *Pfr* grows and produces much more conidia than *B. bassiana* when infecting *B. argentifolii* under controlled environmental conditions [59]. These results indicate the possibility of improving conidia yield by rational design of culture media. Daigle et al. [75] produced aerial conidia of *Pfr* by solid state fermentation on polypropylene bags with 700 g of rice flour. They obtained about 6 x 10^{10} cfu per bag after 2 weeks. AGROBIONSA produces aerial conidia on bags with only 300 g of supplemented rice per bag, yielding 3 x 10^{11} conidia per bag after 2 weeks (AGROBIONSA, unpublished data). Thanks to this achievement, Pae-Sin® is now successfully sold in the Mexican market.

Dry powder of conidia of *Pfr* is highly stable when stored at 4°C. For example, viability of conidia produced by Mycotech was not significantly different after 22 months storage [59]. AGROBIONSA stores conidia mixed with diatomite at 4°C for up to six months before selling it, without loosing efficacy.

Although surface conidiation is being used to produce mycoinsecticides, scale-up may be complicated, especially if the strain's conidiation is light-dependant. By submerged fermentation it is

easier to sacale-up *Pfr* propagule production, and we showed that is possible to increase productivity more than 40 times [74], both by increasing yield of infective propagules, and decreasing production time from 14 d or more to less than 6 d. Submerged fermentation technology is more amenable to scale up, and its possible to have stricter control of all process variables, making feasible to manipulate the resistance and virulence of the propagules [76]. Additionally, in submerged cultivation is possible to obtain high concentrations of toxins to improve bioinsecticide performance.

3.3 Blastospores

Blastospores or "hyphal bodies" are single-cell, thin-walled, "mycelial fragments" which are produced at the hyphal tips and directly from individual blastospores [77, 78]. Although infectious, blastospores are short-lived and do not survive adverse environmental conditions to the same extent as conidia [69]. According to Eyal et al. [67], when formulating the media with complex nitrogen sources, production of mycelium is favoured by complex carbon sources, while using pure sugars such as sucrose or dextrose results in the formation of mainly blastospores. Using the same principle, Jackson et al. [77, 79] achieved 5.8 x 10^8 blastospores·mL^{-1} (80 g·L^{-1} glucose, 13.2 g·L^{-1} casamino acids, plus nine vitamins) from an initial concentration of 5 x 10^4 conidia·mL^{-1}, in a three day fermentation. In this work, authors point out that blastospores are produced during growth, and production might be stopped by the exhaustion of the nitrogen source. In our laboratory we produce more than 3 x 10^8 blastospores·mL^{-1} using a cheaper medium containing only 20 g·L^{-1} glucose, 10 g·L^{-1} casein peptone and 2 g·L^{-1} yeast extract (unpublished data).

Virulence of hyphal bodies is usually considered low compared to aerial conidia [76]. Vandemberg [80] compared the efficacy of aerial conidia of *Pfr* and fresh and dried blastospores against the Russian wheat aphid. He concluded that blastospores prepared fresh, air-dried, or freeze-dried are at least as efficacious as aerial conidia. Fargues et al. [81] compared the pathogenic activity of different propagules of *Pfr*. They found that hyphal bodies were the most pathogenic to *Spodoptera frugiperda*, followed by germinated conidia and finally aerial conidia (61%, 46% and 29% mortality respectively). These laboratory tests, however, were done at high relative humidity and protected from UV exposure, conditions not always reproducible in open field. From the other side, it is hypothesised that the higher virulence of vegetative propagules is caused by the shortest "germination" time. Under field conditions, where free-moisture requirements represent a significant constrain to biocontrol efficacy, rapid germination by liquid culture produced blastospores should further enhance the ability of these propagules to infect and kill silverleaf whitefly and other susceptible insect pests [77].

Formulation of the hyphal bodies is complicated [76]. Blastospores produced by various entomopathogenic fungi are typically larger than aerial conidia, are not amenable to simple drying techniques and tend to perish more rapidly during storage [77]. Overnight air drying of blastospores until 1-5% (w/w) moisture in a biological containment hood (25% to 40% relative air humidity) resulted in good initial viability but poor stability (30% survival at 4°C after only 30 d). Freeze dried vials of 2 mL blastospores suspension containing 10% (w/v) lactose and 1% (w/v) bovine serum albumin and stored in vacuum gave better results: viability was 95% after 30 d and 68% after 150 d when stored at 4°C. Unfortunately blastospore survival fell to 1% after 30 d when stored at 22°C [77]. Stephan and Zimmermann [71] tested spray drying blastospores, using skimmed milk powder and sugar-beet syrup as drying protectors (64 ± 2°C inlet, 48 ± 2°C outlet temperature). Viability of spray-dried submerged spores was between 82.5% and 88.7%, but they do not report stability of dried blastospores. Germination of spray-dried spores was delayed compared with that of freshly harvested submerged spores, and this may reduce the proposed advantage of blastospores over aerial conidia.

A blastospore-based preparation is available in the USA, Canada, Europe, North of Africa and Asia, under the name of PreFeRal® (PFR-97 20%, 10^9 CFU·g^{-1} blastospores) (W. R. Grace & Co., Columbia, Maryland, U.S.A.; Biovest, Westerlo, Belgium) [57, 82, 83]. The USDA-ARS and Eco Soil Systems, Inc. (San Diego, California, U.S.A.)

avoided the blastospore stabilisation problems by offering *Pfr* blastospores produced *in situ*, in portable reactors, as required by the client [84].

3.4 Submerged conidia

Various entomopathogenic fungi produce conidia in submerged culture [85]. Submerged conidia can form the outer layer present on aerial conidia although to a lesser extent [69]. Also they seem less hydrophobic, smaller, and thinner-walled than aerial conidia. That might reduce their adherence to the insect's epicuticle, and resistance to unfavourable conditions, but in the case of submerged conidia of *B. bassiana*, they germinate faster than aerial conidia [69], thus acquiring the advantage of vegetative propagules without completely losing resistance and adherence capabilities.

De la Torre and Cárdenas-Cota [74] first reported conidia production in submerged cultivation of *Pfr* in 1996. Using a simple carbon source, glucose (30 $g \cdot L^{-1}$), and a simple nitrogen source, ammonium nitrate (0.7 $g \cdot L^{-1}$), supplemented with yeast extract (1 $g \cdot L^{-1}$). Submerged conidia concentrations achieved were 10^8 conidia$\cdot mL^{-1}$ in a 6-L fermenter (4-L V_L), maintaining dissolved oxygen over 20% of saturation with air. High C:N is needed to produce submerged conidia. Using the same basic media ("Paris" media) but with a low C:N (10 $g \cdot L^{-1}$ glucose, 5 $g \cdot L^{-1}$ yeast extract), Vidal et al. [78] obtained blastospores. In the same paper, they also reported "conidia-like" structures when culturing *Pfr* in other media of higher C:N designed for the production of conidia of *B. bassiana*. We investigated the effect of C:N and sporulation, using a defined medium with 50 $g \cdot L^{-1}$ of glucose and several salts, changing NH_4NO_3 to modify C:N from 9 to 150. Conidia yield had a maximum at a C:N of 25, but conidia:blastospore ratio continuously increased until it was of 92.5% at a C:N of 150.

Russian researchers reported *B. bassiana* conidia yields of 5 x 10^8 to 1 x 10^9 conidia$\cdot mL^{-1}$ and 2 x 10^9 conidia$\cdot mL^{-1}$ [76]. Conidia yield of *Pfr* in submerged culture is comparatively low, but can be improved manipulating the culture conditions. Other nutrients that play a modest role on conidia yield are phosphate and calcium (Zengguo He and Fernández-Sumano, unpublished results), and many remain to be discovered.

A different approach to improve submerged conidia productivity is through microcycle conidiation, which is possible to induce in *Pfr* by temperature shift [74, 86]. Submerged conidia are produced when cultivated under optimal temperature after a period of incubation at supraoptimal temperatures. With microcycle conidiation may be possible to produce resistant submerged conidia from high blastospore density broth in the future. Although this technology is promising, yet there are many questions to answer before a pesticide formulated with submerged conidia became commercially available.

3.5 Conclusions

Information regarding propagule production of *Pfr* is still scarce, specially that concerning reactor design. Research has been focused mainly on the effect of nutrients over propagule yield and properties. To date, patents have been issued to protect mycelium and blastospore production, and aerial conidia and blastospore-based biopesticides are now commercially available. Compared with stability of aerial conidia simply collected and mixed with diatomite, blastospore conservation is rather costly and disappointing, but this limitation may be ignored if blastospores are produced as required, thanks to their short production time. Because of the convenience of submerged culture, it seems that future research is going to be focused on the factors influencing blastospore and submerged conidia yield, virulence and resistance. Aerial conidia production will continue to be a viable and interesting alternative at least for low-scale production, especially for markets where long shelf life is still a need. Mycelium production is relatively easy, but no products have reached the market after six years from the patent publication, and it is not likely that it receive renewed attention in the years to come.

4 *Steinernema feltiae*

Entomopathogenic nematodes have been successfully used to control a wide range of insects, mainly soil-dwelling larval stages of various Lepidoptera and Coleoptera. No detrimental effects have been shown on non-

targeted species [87, 88]. Most of the entomopathohenic nematode species known are comprised into the *Steinernema* and *Heterorhabditis* genus. Therefore, those genera have been the most studied for biological control purposes.

Steinernematids and heterorhabditids are symbiotically associated to *Xenorhabdus* spp. and *Photorhabdus* spp., respectively [89, 90]. *Xenorhabdus* and *Photorhabdus* are motile Gram-negative bacteria and have phenotypic variant forms. The so-called Phase I is essential for both effective entomopathogenic activity and accomplishment of the worms' life cycle, because Phase I secretes several enzymes and broad spectrum antibiotics [91, 92]. The variant forms, called phase II bacteria, seems to be induced during prolonged cultivation. They are altered in many properties (e.g., lower proteolytic activity) that might result in both inefficient killing of the insect host and nematode propagation.

In nature, infection of the insect host by both *Steinernema* and *Heterorhabditis* is started by the so-called infective juvenile stage (IJ), which is able to survive in the environment for long periods of time. These periods depend on the nematode species and environmental conditions (mainly, temperature and moisture) [93]. The symbiotic bacteria in turn are found in the alimentary tract of the IJ stage. Once a potential host is found by the IJs, it can be invaded through different ways. Steinernematids appear to enter the host's hemocoel through the insect mouth, anus, or spiracles. Additionally, heterorhabditids have a tooth that can be used to directly penetrate the cuticle of the host. The nematodes then release the symbiotic bacteria, which multiply and kill the host within 48 h. Worms ingest bacterial cells and host tissues, developing to adults. Steinernematids are amphimictic (male and female individuals) in all generations. The IJs of heterorhabditids develop into hermaphroditic females, which produce a sexual generation that in turn produces new hermaphroditic IJs [91]. The first adult generation is composed of larger worms than any succeeding generation. The progeny of the second adult generation, finding the nourishment depleted, generates the new IJs. Sometimes a full third generation may be observed depending on the size of the host. Finally, IJs exit the insect cadaver to seek new preys. Therefore, IJ stage mass production is of particular interest for field application.

4.1 Propagation of entomopathogenic nematodes

Entomopathogenic nematodes have been propagated by *in vivo* and *in vitro* cultures (axenic and monoxenic) [94, 95, 96, 97, 98]. Friedman [99] has reviewed different methods for insect-killing nematode production and made comparisons among them based on the process-economy involved. According to Friedman and co-workers [100] and Pace and co-workers [101], the submerged monoxenic culture is the most suitable for the large-scale production of IJ stages, as higher productivity is obtained and process is more easily controlled. Although industrial production of IJs of some nematode species is already done [99, 102, 103, 104], the production processes are still empirical. There is a lack of knowledge required for a better understanding of the whole process. Much of the information concerning the production of entomopathogenic nematodes is found in private reports, so it has not been available to the public [93]. For example, there is no information published about the worm propagation kinetics in axenic and monoxenic cultures nor the oxygen requirements of different stages of the nematodes (there are some data for IJs [105]). Furthermore, there is no data published referring to the effects caused on nematode population by actual hydrodynamics in liquid cultures carried out in agitated reactors, as well as to the convective oxygen transfer rates achieved in such processes.

Production of large amounts of IJ stages requires robust processes for liquid monoxenic propagation of worms. In most of the cases, the reactor represents the heart of the entire process. In this sense, the design of the proper reactor becomes a task of the major importance. It is well known that the global reaction behaviour inside a reactor depends on many important factors. Among them, the following: a) the reaction kinetics involved; b) the mixing forces; and c) the oxygen transfer rate. All of them are affected by the actual hydrodynamics that in turn depend on geometrical factors, operating conditions and some physical properties, like the broth viscosity.

Our research group has been working on the former topics. In the rest part of the chapter-section, relevant results are discussed.

4.1.1 Propagation kinetics in submerged monoxenic culture

This section presents some results concerning the propagation kinetics of *Steinernema feltiae* in presence of its symbiotic bacterium, *Xenorhabdus nematophilus* at the flask level. Table 1 summarises main results obtained using two complex media (medium 1, modified from [100]; medium 2, modified from [98])

Because nematode propagation mechanism is very different from that of binary fission found in unicellular organisms (e.g., bacteria, bacilli), we could not use the existing tools developed for such kinetic analysis. Our group took advantage of the classical kinetics approach so following considerations were done:

Taking into account the limiting conditions for the worm propagation:

$$\left.\begin{matrix} t = 0, & C = C_0 \\ t = \infty, & C = C_F \end{matrix}\right\} \quad (1)$$

Such conditions can be rewritten based on both the dimensionless concentration, χ, and the corrected time, t_C,

$$\left.\begin{matrix} t_C = 0, & \chi = 0 \\ t_C = \infty, & \chi = 1 \end{matrix}\right\} \quad (2)$$

The following equation is proposed as a mathematical model to describe the dependence of χ on t_C,

$$\chi = 1 - EXP(-kt_C) \quad (3)$$

where k is a constant to be determined. Equation 3 can be obtained from the integration of Equation 4 when $C = C_0$ at $t_C = 0$,

$$\frac{dC}{dt_C} = -kC \quad (4)$$

Experimental data were adjusted to Equation 3 by a non-linear regression routine. Figure 1 shows the best fit obtained in the case of medium 2. Obtained values of constant k were 0.43 d^{-1} and 0.51 d^{-1} for media 1 and 2, respectively.

Table 1. Kinetics parameters obtained in the submerged monoxenic culture of *S. feltiae*

Medium	C_0	C_F	k	% of IJ at day 20
1	620	143,000	0.43	99
2	510	205,000	0.51	97

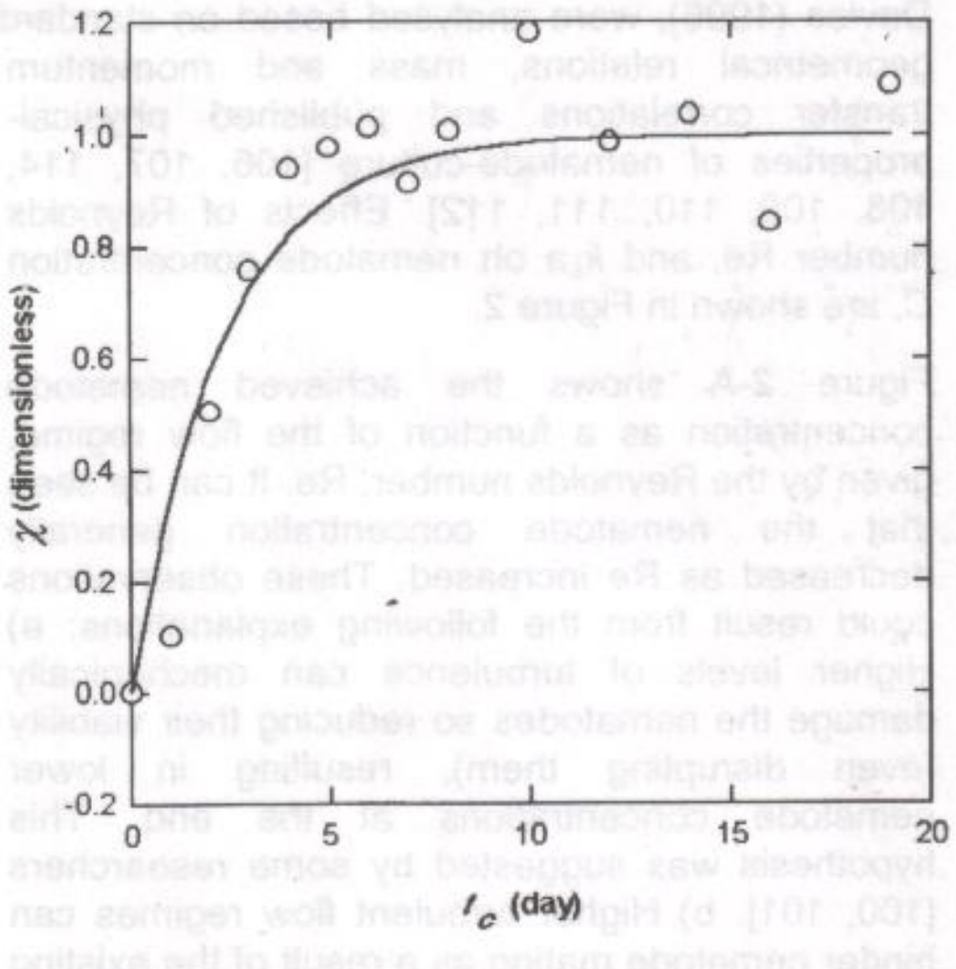

Figure 1. Evolution of dimensionless nematode concentration χ in medium 2. Experimental data, o; Best fit, –.

Media 1 and 2 promoted high nematode concentrations (140,000 to 200,000 per mL) with more than 95% of IJ stages at the culture end and total multiplication factors from 230 to 400 times the initial population. Changes in total nematode population can be modelled using a classical kinetics approach. It shows that very complex biological systems can be reasonably represented using simple models for engineering purposes. Additionaly, the quality of available protein in the culture medium appeared to be of major importance to obtain high multiplication factors.

More investigation is necessary in order to improve culture media formulations.

4.1.2 Hydrodynamics and Oxygen transfer

Results and operating conditions of reactor-level cultures of entomopathogenic nematodes published by Pace and co-workers (1986), Friedman and co-workers (1989) and Surrey and Davies (1996), were analysed based on standard geometrical relations, mass and momentum transfer correlations and published physical-properties of nematode-culture [106, 107, 114, 108, 109, 110, 111, 112]. Effects of Reynolds number Re, and k_La on nematode concentration *C*, are shown in Figure **2**.

Figure **2**-A shows the achieved nematode concentration as a function of the flow regime, given by the Reynolds number, Re. It can be seen that the nematode concentration generally decreased as Re increased. These observations could result from the following explanations: a) Higher levels of turbulence can mechanically damage the nematodes so reducing their viability (even disrupting them), resulting in lower nematode concentrations at the end. This hypothesis was suggested by some researchers [100, 101]. b) Higher turbulent flow regimes can hinder nematode mating as a result of the existing fluid forces. It is known that an effective copulative process needs the nematode male to coil around the female. Such a relation between hydro-dynamics and mating was timidly suggested by Pace and co-workers 15 years ago [101]. Since then, no one has again mentioned this possibility. c) The effect could be due to both reasons.

According to Figure **2**-A, for *S. feltiae* the best results were obtained at Re < 250, yielding an average nematode concentration of 93,000 per mL. Nevertheless, increments in the Reynolds number from 93 to 630, did not affect importantly the achieved heterorhabditidis concentration (110,000 nematodes·mL^{-1}), suggesting that neither viability nor reproduction process of hetero-rhabditidis were affected by actual hydrodynamics in those experiments.

Figure **2**-B presents the dependence of the achieved nematode concentration as a function of the volumetric oxygen transfer coefficient, k_La.

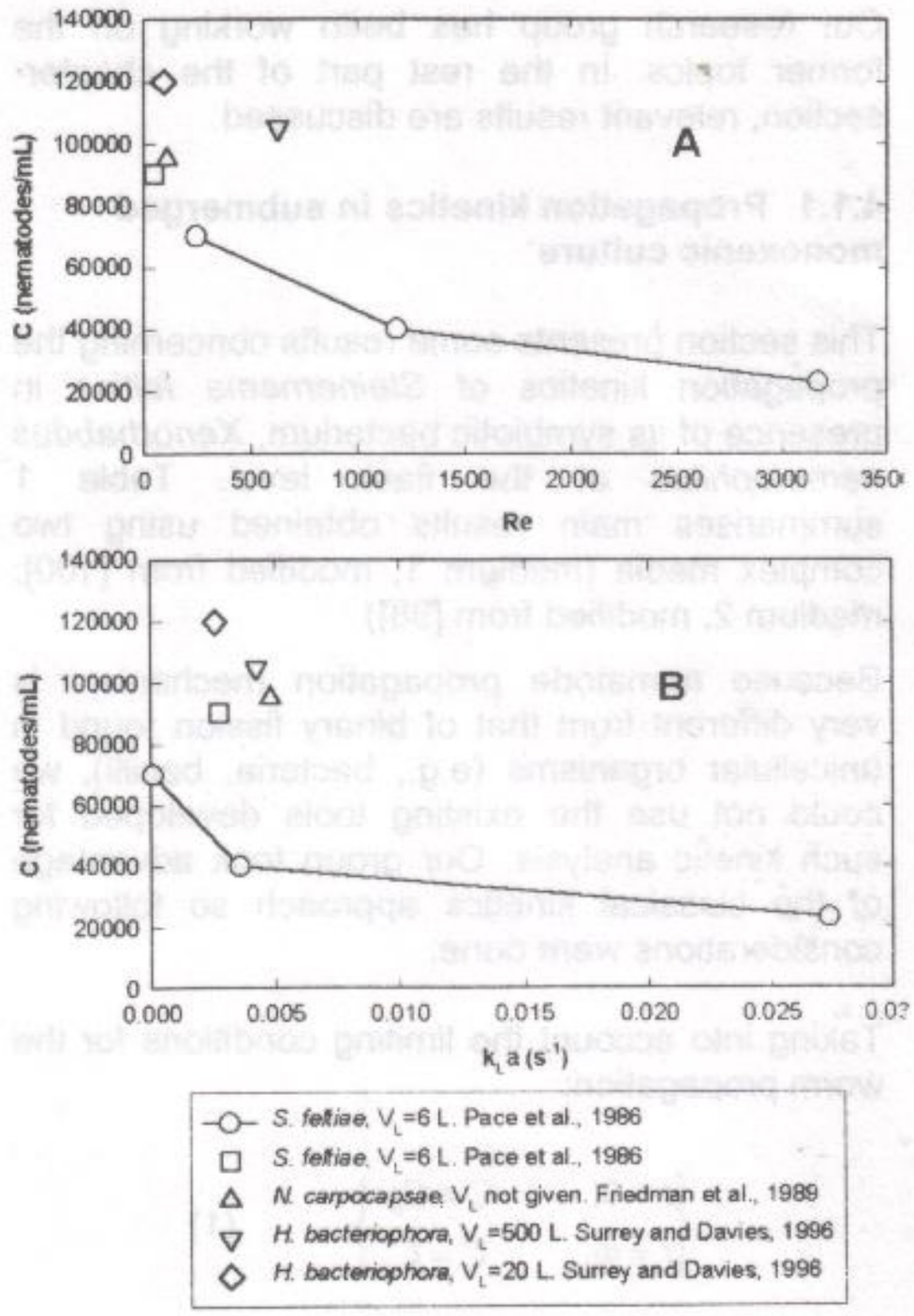

Figure 2. Obtained results based on the information published by different authors. Each point implies one experiment (–○–, stirred tank reactors; □, △, ▽, ◇, pneumatically agitated reactors). A) Influence of the Reynolds number on the achieved nematode concentration. B) Effect of the oxygen transfer coefficient, k_La, on the achieved nematode concentration.

Best results implied an average nematode concentration of 102,000 per mL, and were associated with k_La values from 0.003 to 0.005 s^{-1}. The lowest nematode concentration (23,000 per mL, *S. feltiae*) was associated with both the highest k_La value (k_La = 0.096 s^{-1}), and the highest Reynolds number (Re = 3,173) and was obtained using a stirred tank reactor. It has hypothesised that even oxygen transfer rate was good as a result of higher Reynolds number,

nematode propagation was not very effective because higher turbulence did reduce nematode viability and/or hinder the mating process. Both Figure **2**-A and B, suggest that best operating conditions to propagate entomopathogenic nematodes at reactor level involve Re < 250 and k_La values between 0.003 and 0.005 s^{-1}, if one considers published results. Such best results were obtained in pneumatically agitated reactors.

In order to assess the hydrodynamics effects on the nematode propagation it is recommended to have knowledge on two basic aspects: a) The magnitude of the forces necessary to disrupt one nematode, and how these forces can change with nematode species in turn, developmental stage and nematode age. Such data are unavailable in the literature (few data have been published for hybridomas and individual cells [113]). b) It is necessary to count on a good description of the fluid flow existing within a reactor. Combination of the former aspects can allow one to make a quantitative analysis about the effects on nematode propagation caused by the actual hydrodynamics (reduction of nematode viability or hindering of the mating process).

Data included in Figure **2**, involved average shear rates $\dot{\gamma}_a$, from 10 s^{-1} to 130 s^{-1}. There was no possibility to estimate the shear stress values involved in those experiments, nor authors provided such data.

There are published some empirical data about nematode disruption and nematode viability losses caused by actual hydrodynamics. This information is shown in Table 2.

None of the authors cited in Table 2 give any information about the magnitude of the shear stress τ, nor shear rate or its average value $\dot{\gamma}_a$, involve in their experiments, so it is not possible to define an hydrodynamic condition on a quantitative basis. Therefore, there still remains the question about what is the relation between actual hydrodynamics and nematode viability.

Based on the maximum calculated $\dot{\gamma}_a$ value of 130 s^{-1} considering different nematode propagation systems [101, 100, 98] and the apparent great vulnerability of the 1st generation females to the hydrodynamic conditions, our group carried out shear experiments of nematode suspensions in viscometric fixtures [115].

The highest nematode concentration tested was 30 worms·mL^{-1} with 90% gravid females of 7-10 mm in length. All 1st generation females were

Table 2. Published results about hydrodynamic effects on entomopathogenic nematodes.

Entomopathogenic nematode	Developmental stage	System used	Effects on nematode	Reference
S. feltiae	Adult female, 1st generation	Stirred tank, flat bladed turbine*	Disruption at tip speed velocity [$=\pi dN$], ~ 1 m· s^{-1}	[101]
Not given	Different stages	Concentric cylinders**	Disruption at bob (inner cylinder) velocity: J1 ~3000 rpm J2 ~2800 rpm Adults~1800 rpm	[100]
Different species	IJ	Homogeniser, back pressure 10 barg***	Viability losses after 8 passes: *P. hermaphrodita*, 97% *S. feltiae*, 49% *H. megidis*, 55%	[114]

* Worms suspended in water. Nematode concentration not given.
** No information about neither the sample preparation nor concentration.
*** Mature cultures diluted in water to give 400-700 IJ·mL^{-1}.

viable after shear finished, so worms were capable to resist shear stresses τ, up to 3.2 $N \cdot m^{-2}$. After experiments finished, all nematodes were re-washed, then sanitised and replaced into the original cultures. Death of 30-40% of the sheared nematodes was observed two-three days later. Nevertheless, viable worms continued "normal" development. Such viability losses could be caused by the applied shear, and in some extent, by oxygen limitation and starvation. In viscometric experiments testing nematode-culture broths, we found that only recent J2 stages (at a concentration of 130,000 $J2 \cdot mL^{-1}$) were vulnerable to the applied shear rates (150 to 450 s^{-1}) and shear stresses (2 to 6.6 $N \cdot m^{-2}$), so viability losses of 85% were found after shear ceased. The same conditions did not affect the viability of all other *S. feltiae* stages (even at concentrations of 130,000-150,000 per mL, for the J3-IJ stages). Therefore: a) Actual hydrodynamics can cause viability losses in *S. feltiae* population, even worm disruption, depending on the developmental stage and nematode concentration, but the nematode suspensions must be subjected to $\dot{\gamma}_a$ values considerably higher than 130 s^{-1}, involving τ values of at least 6.6 $N \cdot m^{-2}$. b) Taking into account that most of the *S. feltiae* stages can survive at $\dot{\gamma}_a$ values of 450 s^{-1} in controlled fluid-flow systems, it is reasonable to infer that at reactor-level operating conditions involving $\dot{\gamma}_a$ values from 10 s^{-1} to 130 s^{-1}, low multiplication factors can not result from viability losses due to the hydrodynamic conditions.

Our group has hypothesised that if both the oxygen supply and nourishment are enough for *S. feltiae* propagation, and hydrodynamics involve $\dot{\gamma}_a$ values lower than 130 s^{-1}, low multiplication factors will result because hindering of the mating process, as a result of the acting fluid stream forces.

Based on dimensional analysis, it is well known that for a hydrodynamic system,

$$\mathrm{Re} \propto \frac{LV}{\eta} \quad (5)$$

where L and V are the characteristics length and fluid velocity, respectively. On the other hand, oxygen transfer efficiency has been related to the hydrodynamics as follows

$$k_L a \propto \mathrm{Re} \quad (6)$$

within a variable range of Re.

Liquid monoxenic cultures of *S. feltiae* were done using different bottle diameters. That was done to assess the hydrodynamic effects on worm propagation. Figure 3 presents the dependence of the total multiplication factor, *MF*, on bottle diameter at an orbital shaking velocity of 200 rpm.

None of the hydrodynamic conditions apparently affected nematode's viability compared to the control cultures. In consequence, possible viability losses in the bottle-cultures different from that caused by natural ageing were discarded. Another factors must be involved to explain results contained in Figure 3. Such results might be explained in part on the basis of the oxygen availability.

Although D_2 bottles induce more turbulence than D_1 bottles at the same shaking velocity (according to relation 5), such increments in turbulence seems to be not very important to hinder the mating among worms. On the other hand, $k_L a$

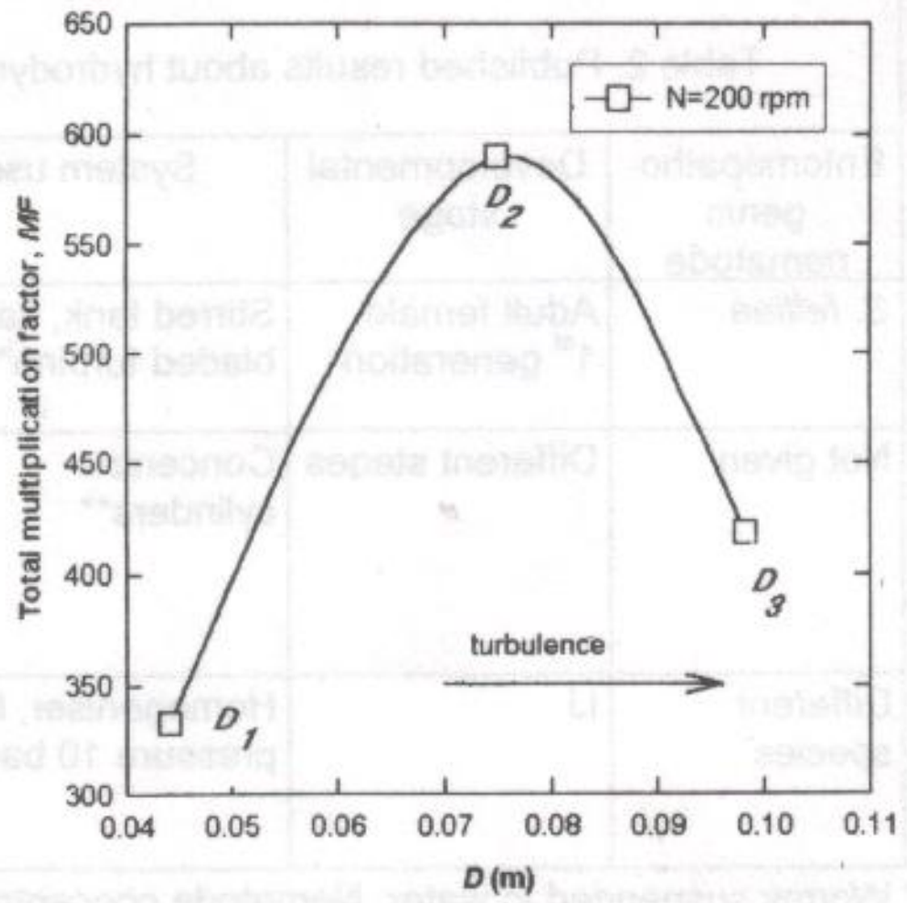

Figure 3. Total multiplication factor dependence on bottle diameter. *S. feltiae* in monoxenic culture.

involved in D_2 bottles could be higher than that in D_1 bottles (according to relation 6). So, without hindering the worm coupling and providing better oxygen supply at the same time, D_2 bottle systems rendered higher nematode concen-trations than D_1 bottles. A similar situation appears to occur between D_1 and D_3 bottles, where achieved nematode concentrations are higher in D_3 bottles due probably because higher oxygen availability in D_3 systems. A different result occurred between D_2 and D_3 bottles. Higher nematode concentrations were achieved in D_2 bottles, even better surface aeration could be encountered in D_3 bottles. This difference could result from more turbulent conditions existing in D_3 bottles in comparison to those in D_2 bottles. According to this, the mating process could be more hindered in D_3 bottles, so resulting in lower final nematode concentrations.

4.2 Conclusions

In order to improve the productivity in *S. feltiae* propagation processes, care must be taken of the mating process that seems to be more sensitive to increments in turbulence than the viability losses caused by the same reasons. Nowadays, our entomopathogenic-nematode subgroup is working on the involved hydrodynamics on a quantitative basis (both flask and reactor scale), and on the oxygen demand of the different developmental nematode stages, and oxygen transfer rates involved in different propagation systems.

4.3 Abbreviations

C, total nematode concentration, nematodes·mL^{-1};
C_O, initial total nematode concentration, nematodes·mL^{-1};
C_F, final total nematode concentration, nematodes·mL^{-1};
D, dilution rate, h^{-1}; tank or flask diameter, m;
k, reaction rate constant, d^{-1};
k_La, gas-liquid mass transfer coefficient, s^{-1};
L, characteristic length, m;
MF, total multiplication factor, (C_F/C_0); dimensionless;
N, orbital shaking velocity, rpm;
Re, Reynolds number, dimensionless;
t, time, d;
t_C, corrected time, $(t - ts)$; d;
ts, time just before nematode population starts growing, d;
V, characteristic velocity, m·s^{-1};
V_L, operating liquid volume, m^3;
χ, dimensionless nematode concentration, $(C-C_0)/(C_F-C_0)$; dimensionless;
$\dot{\gamma}_a$, average shear rate, s^{-1};
η, viscosity, Pa·s;
τ, shear stress, N·m^{-2} (= Pa);

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