BIOTECHNICS WASTEWATER

Biotechnics/ Wastewater

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Controlled Release of Immobilized Cells as a Strategy to Regulate Ecological Competence of Inocula

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The ecological competence (the ability of microbial cells/inocula to compete and survive in nature) of laboratory/bioreactor prepared inocula is paramount to commercial exploitation of biotechnological processes initiated by the addition of microbial cultures to natural habitats. Such processes include waste-treatment, bioremediation, dairy and food, agricultural and environmental systems and are characterized by a general inability to regulate the process environment stringently. Such inocula systems will require, as a first step, an efficient formulation and delivery system, based on microenvironmental control, directed at minimizing the lag period and maximizing competitive advantage to the introduced microorganisms.

The use of polymer gels, for example alginate, to immobilize cells has allowed the development of spatially organized microenvironments with control on the degree of protection afforded, the rate of cell release and the juxtapositioning of cells with nutrients and/or selective agents or chemicals.

The characteristics of the gel and its shape has a major influence on the microniche created. Through the control of the gellation process the rate of diffusion of nutrients and the rate of polymer breakdown (or cell release) can be regulated. Surface area to volume ratio can influence the biomass distribution as can the initial biomass loading. The radial gradient created (or the resulting degree of nutrient limitation) in gel beads can have significant influence on both the distribution and behaviour of the immobilized biomass. Thus the combination of immobilization technology and nutrient limitation has resulted in the creation of microenvironments in both space and time dimensions. The resultant inocula delivery systems improve the resistance of the culture and regulate the release of cells with enhanced resistance to stress which is advantageous when "the window of opportunity" to ensure successful colonization can be restricted.

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1 Introduction

The application of ecological principles will inevitably be necessary for the successful exploitation of some novel biotechnological systems currently characterized by their unstable nature. The limited ability of microbial cells to influence their environment is a major restriction when cultures are exposed to dynamic environmental conditions. Thus an enhancement and understanding of microbial plasticity to interruptions or environmental perturbations is fundamental if those process environments, not amenable to strict control, are to be exploited.

Natural evolutionary pressures and the biotechnologist appear to have developed microorganisms in similar directions each developing the specialist skills or characteristics which in turn affects the general robustness of the cell resulting in the relegation of cell types to narrow niches with ever-decreasing capacities to adapt to a wider range of environments.

Evolution, in nature, of procaryotic systems could not proceed towards the selection of a few 'super-competent generalist organisms' but had to move divergently towards a spread of genetic information among a wide range of more specialist organisms [1]. Thus a high degree of specialization exists in individual organisms with respect to their resistance to a particular variable (e.g. oligotrophic, halophilic, thermophilic, psychrophilic, anaerobic etc.) which results in a characteristic structure for microbial communities in most natural systems.

In practice cultures are generally grown as homogeneous cell suspensions in nutrient rich liquid media under optimum conditions. Natural environments or habitats tend to be more complex with a range of surfaces and interfaces, that influence the microbial population which may be relatively slow growing or dormant, sessile, and exposed to nutrient limitation, often resulting in a heterogeneous distribution of microbial populations throughout the environment.

Considering the energetics of the microbial cell, most optimized commercial treatments or recombinant cultures probably represent an inefficient and undesirable system with the ultimate goal of complete conversion of nutrients into products, often superfluous to the cells requirements. Inherently unstable biotechnological processes will arise when such microbial cells optimized for specialist laboratory or bioreactor conditions are exposed to heterogeneous or open systems containing "fitter" or more robust microbial populations with selective or enhanced ability to respond to dynamic conditions.

2 Ecological Competence and Stability of Commercial Inocula

The ecological competence of artificially prepared inocula is paramount to commercial exploitation of biotechnological processes initiated by adding microbial cultures to natural habitats. These include waste-treatment, bioremediation, dairy and food, agricultural and environmental systems. Such processes are characterized by an inability to regulate stringently the process environment.

Judging from the inconsistency of, for example, biological control reported in the literature [2–5] there appear to be major problems in the development of the technology especially in relation to the stability of the microbial system employed.

The metabolic activity and adaptability or ecological competence of commercial inoculants appears to be a major limitation. For example, Nesbakken and Broch-Due (6) suggested that the inefficacy of many commercial inoculants, used in the ensilement of forage crops, arose from lack of sufficient numbers of bacteria. Such inoculants are added as freeze-dried powder, or immediately after suspension of freeze-dried cultures in water, resulting in insufficient numbers of metabolically active cells – inoculants cultured overnight before application resulted in improved activity. They also highlight that many commercial inoculants contain cultures that are not adapted to forages.

Commercial microbial inocula will require a high degree of robustness if exposed to diverse environments. Technologies based on the addition of inocula to what are basically natural habitats must consider, for example, that with very few exceptions, these habitats are heterogeneous. This heterogeneity may include discontinuity of flow, gas/liquid/solid interfaces, temperature and pH variations, availability of both simple and complex substrates, multiple nutrient limitations and competition with indigenous microbial populations. Further heterogeneity may arise due to the release of solutes from particulate organic matter or complex polymers which in turn may depend on competing microbial enzyme activities. Such degradation processes can result in chemical and physical gradients leading to a range of microenvironments that ultimately gives rise to transient states in the metabolic functioning of the cells.

Environments may also range from (a) the original ecological niche from which the culture was first isolated, (b) the bioreactor used to grow the inoculum, (c) those conditions pertaining during unit operations associated with harvesting, storage, transport and delivery of the inoculum to its ultimate site of action, (d) competition and predation from indigenous microflora. In addition the exploitation of microbial inocula will depend on commercial product parameters such as the convenience and ease of handling. Ideally it should be possible to bulk produce the inoculum and also handle and store it like a chemical.

Likely successful strategies to overcome the changing or dynamic environmental conditions would appear to be based on some form of spatial organiza-

tion involving: (a) Protection–through the creation of micro-environments, providing the desired conditions or populations and the physical regulatory systems, to minimize the effect of fluctuations in the macroenvironment. Protection from competition, predation and lysis would also be important. (b) Controlled release – through the creation of niches or microenvironments which assist the cells to adapt to the new environmental conditions and then release the adapted cells under controlled conditions.

3 Motility, Juxtapositioning and Spatial Organization

Studies on microbial ecology have demonstrated that spatial organization is a fundamental process in microbial ecosystems. At the population level cells respond to environmental changes by altering their spatial relationships. Such strategies may be necessary when, for example, nutrients are not completely mixed or distributed throughout the environment. An example would be the addition of rhizobia inocula to soil – in order to proliferate a symbiotic association with plant roots. Thus the initial step in successful population survival must be to find a host root system.

To overcome such nutrient gradients a fundamental property would appear to be the ability to distribute effectively or move from one location to another. However microorganisms have certain limitations based on their method of locomotion and their method of sensing.

It has been observed that, for example, bacterial cells move with intent, accumulating in regions which are of favourable chemical composition or have suitable environmental conditions [7]. However due to their unicellular nature they can simply accumulate (through rapid cell division) by remaining associated with a food source. Lauffenburger et al. [8] considered the exploitation of such chemotactic response in biotechnological processes and its value in the potential control of microbial population dynamics in non-mixed systems.

It is tempting to assume that as animals move in order to hunt for food consequently the objective for microbial movement is similar, however, such a simplistic analysis ignores the rheological properties of liquid phases relative to microbial cells. Movement for various types of organisms can be fundamentally different, for example, an animal can swim by pushing fluid backward against the inertia of the fluid, momentum is conserved which results in forward motion. This reciprocal type motion is not effective when swimming at low Reynolds number (Nre) hence the "flexible oar" or flagella type motion which is associated with bacteria (an analogous low Nre situation would be an animal swimming in mud or molasses). In most natural environments bacterial motility is associated with low Nre, consequently cells cannot easily escape from their environment and are prisoners of the bulk of the fluid phases.

Bacterial motility is influenced by a variety of environmental stimuli. It is significant that often the stimulus is first transduced by the general physiology of the cell rather than detected by a specialised sensory receptor. Thus energy state becomes a basic sensory input. Taking account of the single cell nature of bacteria, the form of growth, and cell energetics it is most unlikely that microbial systems are designed to "hunt" for food, it is more likely that chemotactic responses and motility are designed to prevent cells from becoming dissociated from existing food sources and thus impose a spatial order based on food supply.

Convection will distribute the bacterial population throughout the bulk fluid or macroenvironment much more efficiently than bacterial motility. The impressive relative speed of a bacterial cell (20–30 cell lengths per s) compared with that of fast animals such as the cheetah (4 body lengths per s) suggests a highly mobile organism. However because of its microscopic size it is simply embarking on a microscopic journey despite its impressive relative speed. Effectively, transport of nutrients or wastes is controlled by diffusion. For example, to increase its food supply by 10% the cell would have to move at a speed of 20 times that which has been observed [9]. Simply, microbial cells cannot move fast enough to overcome the speed or rate of diffusion of small molecular weight nutrients [10, 11].

The nature of cell movement probably further indicates its major function as a sensory mechanism. Bacterial cells move in two phases. First the cell moves in a relatively straight pathway which is then altered to the second phase in which the cell tumbles or alternates its orientation randomly to a new direction. Brown and Berg [12] developed model systems to describe the effects of substrate concentration on the first phase which depends on chemical concentration – when high the path length is extended. Thus in a gradient there is a bias resulting in a drift towards the nutrients. It appears that the cell interprets a spatial gradient as a temporal one – the cell moving in the gradient will sense an increase/decrease in nutrient concentration with time. Microbial cells, due to their small size, are unlikely to identify gradients by sensing the concentration along the length or width of the stationary cell. Consequently the cell moves in order to experience or sense the environment.

A number of studies confirm the sensory nature of microbial movement. Adler [13, 14], Adler and Templeton [15] and Adler et al. [16] studied the responses of *Escherichia coli* to spatial gradients and established a quantitative dose-response curve. These studies showed that (1) chemicals that are extensively metabolized need not necessarily attract, (2) chemicals that are not metabolized may attract, (3) transport of a chemical into the cell is neither necessary nor sufficient for it to attract. These results probably reflects the fact that such cells are sensing an environment likely to provide suitable nutrients rather than simply responding to specific chemicals and highlights the need for a holistic approach to understand the ecological significance of cell movement.

Thus motility in the microbial world is probably not primarily associated with dissemination of cells or seeking out new food sources but rather is directed

towards a bias in favour of remaining associated with (or "immobilized" within) suitable environments when encountered. The balance between cells becoming dissociated from their food source (due to bulk flow of the liquid phase) and remaining associated with the food source (due to motility or chemotactic response) probably represents a slow release mechanism which allows populations to continuously test their environment and to distribute when conditions are favourable.

The activity, dissemination and distribution of cells throughout an environment probably is more dependent on the improved ecological competence arising from the ability to sense, create and exploit microenvironments than from motility directly. Gannon et al. [17] have examined a number of bacterial strains with respect to their ability to move with water through soil. The presence of flagella did not correlate with transport whereas retention was statistically related to size. This study highlights that a number of interacting characters determine whether organisms are transported. Thus the distribution of cells, arising from inocula, throughout an environment such as soil is probably mainly influenced by the ecological competence of the cell to survive the various phases or environmental conditions encountered.

Movement can thus result in a spatial heterogeneity or juxtapositioning of cells within ecosystems. In natural ecosystems microbial cells often form spatial organized ecosystems that act as protective microniches. These "buffer zones" or microenvironments that interface between the microbial cell and the macroenvironment may include surface growth, film formation or floc formation. An essential aspect of these strategies appears to be the fixing or immobilization of a large proportion of the population resulting in the microbial cells being physically confined or localized, often in a polymer matrix, in a certain defined region or space. The influence of such structures on the regulation of diffusion to and from the immobilized cells must be a significant mechanism in protection.

This spatial organization appears to be a natural microbial strategy to overcome changing environments. It is also recognized as a basic ecological principle in both plant and animal population ecology – Allee's principle relating the degree of aggregation and overall densities that result in optimum population growth and survival [18]. For example, clumps of plants or animal herds demonstrate both the competitive and cooperative aspects – too many members exhaust the food resource but an appropriate sized group enhances overall survival.

To date, based on the number of papers published, the major interest in the application of immobilization technology is in the area of process intensification such as the opportunity to convert the traditional batch process to the higher rate continuous one. Consequently advantages cited for immobilized cell versus free cell systems include: (a) reduction in the cost of bioprocessing due to repeated use of biocatalyst, (b) the maintenance of higher cell densities and (c) the provision of a system providing minimal cost for separation.

Relatively less attention has been focussed on the unique effects of immobilization on microbial physiology – often the fact that immobilized cells behaved

differently from free cells has been considered to be a disadvantage. An obvious benefit of immobilized cell technology is in the control and exploitation of the unique microenvironment (especially that associated with gels) created in such systems, specifically, the potential of this microenvironment in the stabilization of microbial cultures and enzymes [19].

The high level of process control that may be exerted on such microenvironment offers special advantages. Effectively it means that in the case of gel bead immobilization one has the potential to introduce numerous "microreactors" into the macroenvironment.

4 Application of Spatial Organization and Microenvironments in the Stabilization of Bioprocesses

Two examples of immobilization leading to spatial organization or juxtapositioning relevant in biotechnological processes can be seen in the exploitation of:

— Natural flocculation mechanisms used to create microenvironments resulting in stabilized microbial processes such as those found in wastewater treatment systems.

— Immobilization techniques used to create microenvironments that enhance inoculum viability.

4.1 Flocculation

Single microbial cells, for example, can respond to stress such as nutrient limitation by: (a) movement or motility based on trophism, towards a suitable microenvironment. (b) altering single cell state to that of flocs or aggregates resulting in the formation of microenvironments consisting of consortia of immobilized cells.

Activated sludge flocs (involving mixed populations) and yeast flocculation (usually involving only one yeast type as in brewing) are examples of spatially organized ecosystems that are controlled by process parameters. However many studies have concentrated on the physical aspects such as settling properties, floc strength etc. [20–23] rather than the physiological ones. These studies do, however, give some insight into the ecological significance of flocculation and especially the microenvironments created.

Spatial organization such as flocculation appears to be a response to starvation or physiological state [24, 25, 26, 27] and both enhance food utilization and provide a degree of protection to cells. The juxtapositioning arising from aggregation of discrete microbial cells may result in complex metabolic interactions.

Jones et al. [28] demonstrated model systems of defined immobilized consortia and their ability to carry out the biogenesis of methane. Conrad et al. [29] described a specialized metabolic function for cellular aggregation or floc formation in anaerobic digestion systems. The juxtapositioning of microbial species resulted in a spatial organization of syntrophic metabolism [30, 31].

Floc structure enhances utilization of substrates in nutrient limited conditions. Complex substrates such as proteins are concentrated in the vicinity of the biomass [32]. The enzyme activity required for substrate breakdown is mainly cell bound [33, 34]. This juxtapositioning of substrate, enzyme activity and biomass confers ecological advantage in competition for breakdown products.

Flocculation also provides a more efficient utilization of nutrients in that any product leaked or released from a cell can be utilized by a neighbouring one. Cell processes, for example wall synthesis, can result in significant leakage occurring, indeed it is postulated that 50% of cell wall material can be turned over per generation [35]. Studies on *E. coli* have shown that peptides released from peptidoglycan can be used as precursors to form new wall material [36, 37].

In microbial flocs the leaked products of one type of microorganism may become the nutrients of another, for example, algal cells leak organic matter which can be used as a concentrating mechanism by bacterial populations. Azam and Ammerman [38] have proposed a microbial interaction whereby the chemotactic abilities and properties of bacterial cells allow them to sense and utilize higher nutrient concentration regions around algal cells. Jackson [39] examined the significance of the size of phytoplankton aggregates or flocs in relation to leakage. If leakage occurs from cells at a constant rate then smaller spatial organizations or flocs will have lower concentrations surrounding them compared with larger flocs. As the biomass contained within a spherical floc increases approximately as the square of the radius the concentration of the leaked material will decrease faster with increasing distance from a small floc than from a larger one.

Spatial organization (such as flocculation) resulting in juxtapositioning provides a mechanism directed at closing the system so that material lost from one cell can be efficiently captured by neighbouring ones. Thus material lost due to death and lysis can be readily utilized by neighbouring cells. In natural populations death and growth are complementary processes and there is little doubt that death and lysis are fundamental parts of the process of growth and survival within such microenvironments [40, 41].

Spatial organization based on flocculation or aggregation appears to be a means of protection during adverse environmental conditions. During starvation a fraction of the population in the centre portion of the floc will be protected and survival will be enhanced as some of the cells lyse and release nutrients that are easily recycled. Thus a fraction of the population is sacrificed so that some survive.

Direct observations on the interior of, for example, activated sludge flocs indicate an abundant presence of extracellular polymers in amorphous forms

surrounding microorganisms in most of the flocs. Thus polymers form a matrix to connect most of the microorganisms together and to maintain the integrity of the flocs through cell immobilization.

Extracellular polymers whether originating from lytic activity or biological synthesis and excretion have been detected on, for example, activated sludge surfaces [27]. A number of investigators have proposed that activated sludge flocs are formed by flocculation of bacteria with naturally occurring polymers acting as floc agents. Tenney and Stumm [42] proposed that biological flocculation occurs through the formation of bridges between cells where naturally occurring polyelectrolytes serve as the bridging polymer. Busch and Stumm [43] showed that polymers extracted from bacterial culture by centrifugation were capable of interparticle bridging. A role for polymers released from cells through lysis has been suggested by Pavoni et al. [27] and Nishikawa and Kuriyama [44] and the polyelectrolyte nature has been shown to enhance flocculation of both sludge and bacterial cultures [45].

Production of extracellular polymers is a common phenomenon in other ecosystems also, for example, most soil microorganisms produce polymers [46] and a number of functions, relating to ecological competence, have been associated with such polymers. These include adhesion, protection against predation and dessication [47–49]. Martens and Frankenberger [50] suggested that polymers, produced by soil bacteria, may also have a function in enhancing enzymatic stability. Microorganisms may retain their extracellular enzymes within these matrices and possibly protect the proteins from breakdown. Such protection would allow an increased exploitation of nutrients in the soil environment.

One of the disadvantages encountered in studies on spatial organization and microenvironments in natural systems has been the number of interacting variables resulting in difficulty in defining, for example, floc structures [21] and in controlling parameters [51, 52]. However the development of immobilization technology, especially the use of natural polymers in gel formation has allowed some degree of process control over spatial organization.

4.2 Immobilization of Inocula

In developing inocula for industrial, agricultural or environmental systems it is essential, in order to stabilize the process and minimize the lag period, to transfer a viable, competitive microbial culture from the inoculum production unit to the process unit. In industrial systems with a high level of process control, it is possible to achieve this through matching the conditions in each unit and then minimizing the transfer time. However in less-controlled biological systems especially those associated with food, agricultural or environmental processes the aseptic conditions of the pure culture inoculum unit contrasts with the complex interacting microbial populations present in, for example, forages, soils