Current state of high-density fermentation technology for prokaryotes cultivation

Research Project 2

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Abstract

Due to the high impact fermentation has in industry as environmentally friendly alternative for biomolecule production, bioprocesses that exhibit improvements in efficiency are needed. The production of biomolecules is related to biomass generation, and therefore great attention has been placed on the investigation of different aspects regarding fermentation in order to increase biomass concentration until high cell density values. Different approaches can be considered for achieving this goal depending on the type microorganism and the product which are wanted to be cultivated and produced. Here, some considerations on prokaryotes fermentation will be referred regarding bioreactor design, medium improvement, operational modules among other aspects for the development of success HDF operations.

1. Introduction

Cell culture and the metabolic activity related to it have been an important aspect of the daily life in humankind since ancient times when fermentation was used for the production of alcoholic beverage and food. Nowadays, different methodologies have served to help us elucidate the nature of processes that take place during fermentation, either at microbiological level as well as biochemical. In addition, advances in technology allow us to maintain certain control over critical parameters during biotechnological operations in order to improve production of biomolecules. Upgrading these aspects have been crucial for taking fermentation processes into large-scale business and creating a new industry based on the ability to produce molecules from living-organism's cultures and supported by strategies that permit lowering costs of productivity and reducing operation time.

Moreover, the emergence of recombinant proteins has brought the possibility to express and produce proteinaceous compounds in hosts that are not the natural producers. This technology is named heterologous expression of proteins. *E. coli* is the microorganism which have been used most extensively for this purpose as it is capable to exhibit high volumetric yields[1], its doubling time is relatively low rendering short fermentation times and it can grow in complex media. However, there are some limiting factors that persist and can hinder the volumetric productivity like dissolved oxygen concentration, availability of carbon and nitrogen sources during fermentation and lack of complete carbon source oxidation that ensures production of organic acids which inhibits bacterial growth[2,3].

In general, production of biomass has been stated as one of the most important parameters to have into account when it comes to high productivity of metabolites and proteins, especially if they are associated to bacterial growth [4]. Different techniques and methodologies have been reported to date in order to avoid or promote the detrimental or enhancing effects that limiting factors exhibit on growth, resulting in that is known as high density fermentation (HDF). This essay is intended to shed light on the current state of different methodologies that have been used to achieve high density fermentation of bacteria and archaeon microorganisms which means that it will not cover advances in HDF of eukaryotic models.

2. High Density Fermentation (HDF)

Under standard fermentation conditions, feeding is only performed once, at the beginning of the process, while other parameters are not thoroughly controlled during the rest of the process. This way, a typical bacterial growth curve is obtained which comprises lag or adaption phase, followed by an exponential phase in which the maximum value for growth rate is achieved, and finally the stationary phase that describes the moment in which mortality rate and growth rate become equal. HDF comprises biotechnological processes in which biomass production of a pertinent microorganism achieves values above those accomplished under conventional growth conditions or typical batch conditions [5]. This can be achieved by controlling different parameters during the fermentation as previously mentioned. Furthermore, with the possibility to obtain higher volumetric productivity (i.e. product or biomass content per volume per time) than usual, it has been

developed, in industry, alternatives in which volume of fermentations are reduced with the aim to obtain the same amount of biomass that can be achieved in higher-sized operations. This is particularly useful in biomolecule manufacturing due to the reduction in cost of operations like production, downstream processes and other aspects related to the fermentation itself[1,6]. Besides, the term HDF is relative to the microorganism as the growth rate and optimal culture conditions differ among species and even strains. Therefore, biomass values ranging from 20 g/L to 50 g/L of cell dry weight have been referred as HDF but also biomass that comprises up to 200 g/L and 300 g/L , so that a specific rang of biomass production considered HDF is difficult to stablish[7,8,5,9].

3. Aerobes

One of the most important limiting factors in aerobic fermentation is oxygen concentration besides the rest of the nutrients. It is well known that under normal conditions, oxygen depletion takes place as it is consumed in the respiratory chain. In these cases, oxygen supply settings become crucial for growth of aerobes as the lack of oxygen or localized anaerobic microenvironments promotes the partial oxidation of carbon sources[3]. Partial oxidation of carbon sources renders into the production of organic acids and the bacterial growth is inhibited as consequence. The most remarkable examples of this kind of inhibition is exhibited by *E. coli* and *Bacillus spp* which produce acetic acid and propionic acid respectively, which after certain concentration they become toxic for the bacteria [4,9].

In general, HDF can be achieved mainly by adjusting the feeding method used in batch production as well. Usually, microbial growth depends mostly on concentration of substrates. With substrate depletion, the growth rate starts to decay. In order to avoid this phenomenon, high concentration of substrates is important to maintain growth rate at maximum all along the process. However, microorganisms can suffer of substrate inhibition when they are in high concentration at the beginning of the fermentation. One way to tackle this drawback is setting a continuous or intermittently feeding system that allows to keep the fermentation under optimal substrate concentration, extending the exponential phase which results in higher values of biomass. This method is very well-known as Fed-batch system. The simplest version of the fed-batch system comprises the total or partial removal of biomass at the end of the process too. In case biomass is not entirely removed, the remaining biomass is used for inoculation of the next batch run. In fed-batch cultures also the volume is increased as the external media is continuously supplied and therefore the substrate content in the external media must be properly adjusted to high concentration in order to avoid underfeeding the culture by substrate dilution. Furthermore, increasing the culture volume also slowdown the microbial growth rate despite the biomass obtained at the end of the process is higher than under standard conditions. This issue has been addressed by performing a stepwise feeding rate in which the limiting-substrate supply is increased depending of the growth phase instead of setting a constant feeding. This technique has proven to be effective on biomass and heterologous expression of proteins in order to achieve HDF values [9].

Some research has been conducted on the possibility of using complex media for successfully obtaining HDF, however, the lack of knowledge on their composition is not recommended for large-scale processes as the components vary in concentration from batch to batch, making difficult to control these parameters. Moreover, another issue to consider is the presence of insoluble compounds or the formation of potential insoluble molecules which might hinder the downstream processes. Some of these compounds consist in insoluble forms of salts which also complicates biomass and mineral concentration measurements [9].

Another very interesting approach in which high volumetric productivity is obtained is the use of two-stage cyclic fed-batch fermentation. Despite this technique is usually more useful for metabolites and proteins than for biomass production, HDF values can be achieved as well. This methodology consists in using two different vessels: the growth and the induction vessel. Most of the biomass is transferred from the growth vessel into the induction compartment after the microorganism is grown up to appropriate levels in order to perform the production of the molecule of interest in a different chamber. The remaining biomass in the growth reactor is used as inoculum for the upcoming batch by adding new fresh media into it while the production process and downstream are carried out separately which makes these two operations easier. This methodology allows continuous batch production as there is no need to entirely stop the operation [9].

Additionally, production of inhibitors is an aspect of high consideration in fermentative operations. Undesired side-products can be generated as consequence of metabolic activity and their accumulation is in most of cases detrimental for microbial growing. In order to maintain low levels of these inhibitors, dialysis has proven to be an adequate strategy to remove these molecules from cultures. Moreover, two different configurations of this dialysis-based strategy have been proposed, from which one has been successfully scaled up to industrial levels: the use of an external dialysis device that connects the fermentation reactor to the reservoir vessel and that showed a significant increase in biomass production in a 300 L- bioreactor reaching HDF values of 190 g/L. The second dialysis-based configuration corresponds to the compartmentalization of one reactor into two by placing a membrane which, however, might hinder stirring operation as the latter can suffer of mechanical stress and limitations in oxygen distribution making it not suitable for large-scale operations. In both cases, the same principle is utilized and consists in the diffusion of certain molecules, in this case the inhibitors, through a semi-permeable membrane from the most concentrated solution to the reservoir.

On the other hand, biomass production is also physical parameter- dependent and can be controlled by the temperature profile that is applied during fermentation as well. This methodology is called temperature-limited fed-batch reactor and might result in less cell mortality, proteolysis and thermolysis which can render high values of biomass and protein production. Besides, the solubility of biomolecules is benefited as proteins and enzymes under high temperatures might result in inclusion bodies if temperature is not properly controlled. In addition, temperature tends to increase in fermentation as a result of the metabolic activity while biomass generation increase the viscosity of the culture that complicates heat transfer. These two aspects are crucial to consider

when designing the operational system as it has been demonstrated that temperature reduction by stablishing thermal profiles stimulates protein solubility. Besides, bacterial cells can suffer of mechanical stress under production as a result of agitation excess. Agitation is performed with the aim of increasing dissolved oxygen as well as for homogeneous substrate distribution. However, under HDF conditions, the augmented viscosity of the culture allows overfed zones and others substrate-scarce which is detrimental for the overall performance of the process. Increasing agitation can be seen as the obvious solution but it can comprise lysis by mechanical stress and foam formation which are undesirable as well[9].

Regarding oxygen availability which turns out to be critical in HDF, pressurized bioreactors and control in gas flow rate have been proposed and demonstrated to increase the dissolve oxygen in vessels as well as to avoid inhibition by high concentrations of dissolved carbon dioxide and organic acid generation. Oxygen itself possess a very limited solubility under standard conditions (i.e. one atmosphere of pressure and room temperature), which makes difficult to maintain suitable concentrations in the liquid phase in HDF[10]. Although oxygen supply can be programmed constant, fermentations might need an increase in air flow rate or even pure oxygen gas supply. Besides, adjustments can be performed on the design of the vessel in which impellers, dimensions and agitation play a significant role for suitable oxygen distribution. Injection of pure oxygen has proven to be useful when it comes to reach HDF values, more precisely 2-11-fold of biomass values reached under air-sparged systems. For its part, fermentations performed in pressurized bioreactors have allowed to obtain around 130 g/L of non-recombinant *E.coli* while a pure-oxygen-supplied process achieved 50 g/L of *Brevibacterium lenins* which exceeded not only the biomass obtained but also the volumetric productivity under standard fed-batch cultivation. Several other parameters related to oxygen and gas composition have been utilized for improving biomass production, for instance, Respiratory Quotient which relates the carbon dioxide generation rate and the oxygen consumption rate[1,9].

As mentioned earlier in this essay, operational control is important in manufacture of biomolecules in order to improve volumetric productivity and yields. The method of choice for tracking specific parameters can be direct and indirect, and they evaluate different aspects that are conducted in fermentations. Indirect control comprises the measurement of certain properties closely related to the parameter that is wanted to be measure, for example, concentration of dissolved oxygen which is directly linked to cell growth as well as calorimetry and pH measurements which are suitable ways to get an idea of the metabolic state of the process. Contrarily, direct control utilizes tools capable of measuring the variable or the compound of concern. However, there are still many molecules which concentrations cannot be measured by using current tools. These methodologies for parameter tracking are usually coupled with feeding modules that regulate and control the supply of substrates or other compounds in order to ensure the parameters are not out of the stablished specifications. Multiple-level systems are commonly applied to biotechnological operations according to culture needs. This way, different parameters can be tested and adjusted depending on the fermentation phase as distinct phases exhibit different limiting factors. For instance, at the beginning of fermentation, the limiting factor for the microbial growth is the concentration of

substrate while at the late exponential phase, the oxygen transfer becomes the limiting factor due to the change in viscosity in the fluid. Additionally, feeding system adjustments are not the only resource with which one can count on to have a proper control over the general operation of fermentations but also genetic content of the strain in used; over the years, genetic manipulation of microorganisms has allowed researchers and companies to develop strains that are resistant to specific detrimental conditions which is useful regarding to HDF. This strategy is usually based on DNA shuffling, directed evolution and synthetic biology. One clear example is the genetic manipulation of lactic acid bacteria, which tend to produce large amounts of organic acids as a result of their metabolic activity, that might result in inhibition. By adapting *Lactobacillus casei* for low pH resistance, 60% higher biomass has been achieved reaching HDF values when the pH drops down to 4.3[9].

One very handy technique for parameter controlling is online tracking. A variety of systems have been developed in order to check different variables of fermentations in real time without interfering with the ongoing process. Mathematical models and sensors must be properly adjusted for reliable monitoring of the specific parameters. Online monitoring has been developed for respiratory quotient, substrate supply, calorimetry measurements and carbon dioxide production rate and has been proven for the development of HDF[9].

4. Anaerobes

Contrarily to fermentation of aerobic microorganisms, anaerobic processes are usually carried out in consortia for anaerobic digestion of waste organic matter or renewable resources. There are not many reports of microorganism-specific anaerobic high cell density fermentations but those with at least formulation of two species of bacteria as the diversity in metabolic activity from different microorganisms is applied to degrade complex media. To matter of this essay, production processes involving specific single species of bacteria will be cover.

Usually, anaerobes grow at lower growth rate than aerobic bacteria and the fact that they produce large amount of organic acids, more than aerobic bacteria stablishes the limitation when it comes to anaerobic fermentations. Moreover, anaerobic microorganisms are frequently used in bioindustry to actually produce propionic acid, acetic acid, succinic acid, among others, as well as molecules used in the energy industry like methane and molecular hydrogen[11,12]. Similarly to aerobes, these organic acids cause inhibition of bacterial growth for anaerobes which is particularly detrimental when it comes to high cell density operations [12].

One of the most important molecules produced in industry by anaerobic fermentation of a single species is propionic acid. Given that microbiological methods to obtain this product are more environmental-friendly than the traditional petroleum-based production, the species *Propionibacterium acidipropionici* has served to properly fulfil this goal. It is used very frequently a recycling-methodology in which biomass is re-used for several continuous or sequential batch production in order to achieve HDF values. With these strategies, up to 100 g/L of biomass have been obtained along with the highest value of volumetric productivity of 14 g/L h despite the final

propionic acid concentration was very low. Furthermore, cell-recycling is performed by ultrafiltration, immobilization, centrifugation or mediated by spin filters. With of the aim of maintaining anaerobic conditions, the sparging must be done with gas composed of either molecular nitrogen, carbon dioxide, or a mix of them [13,14,15]. On the other hand, immobilization of bacteria can also be performed in order to reach HDF levels. Some reports in which *P. acidipropionici* has been adsorbed to different positively charged matrices reveal that higher values of biomass can be obtained by growing bacteria in biofilms in system like that shown in figure 1. The matrices in which these processes are carried out must be pre-treated in some cases in order to confer them the suitable electrochemical properties which allow cell adsorption. In addition, it is important to consider the surface area that the matrix possess and the diameter of the pore depending on the nature of the desired product. For instance, it has been reported that Poraver beads matrix pre-treated with polyethylenimine was 31 times better for *P. acidipropionici* biomass production than Luffa matrix also pre-treated with the same compound while the latter presented 14-fold improvement in propionic acid production. The reason for this is related with the small pore that Poraver possess which allows to achieve bigger cell aggregations hindering substrate diffusion to the inner cells[16].

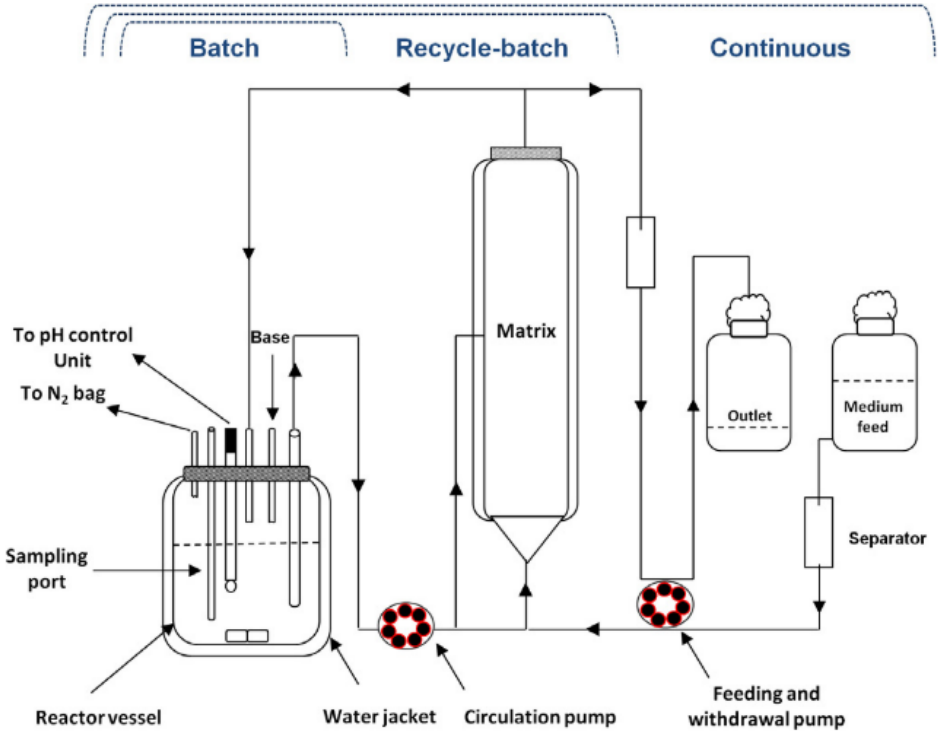

Fig 1. Bioreactor assembly for immobilized P. acidipropionici fermentation using Poraver beads and Luffa matrix. Three different operational configurations are presented in which the single reactor vessel is used for batch production while the latter coupled with the matrix reactor comprise the recycle batch where the media is recirculated. Finally, a continuous fermentation in which the media is constantly supplied

In addition, nylon beads made of polyamide 2200 using 3D printing technology have been reported to be used for *P. acidipropionici* immobilization. This material had to be previously treated with polycation PEI in order to increase interaction with cell surface and thus enhanced biomass production although several immobilization steps must be performed to overcome PEI toxicity to cells[17]. Moreover, fibrous-bed bioreactors (FBB) have been used for immobilization of *P. acidipropionici* as well. These bioreactors comprise containers packed with spiral wound cotton towel which serves as cell support for immobilization. Results with this particular system has elucidated the tight correlation between biomass concentration and butyric and propionic acid production, besides it facilitates the adaptability of microorganisms to high concentrations of organic acids and resistance to contamination when HDF values are reached[18,19,20]. Figure 2 shows a developed system for *P. acidipropionici* immobilization using FBB in which bacteria was grown up to specific biomass values in a stirred tank reactor connected for further immobilization into the spiral wound cotton packed column[21]

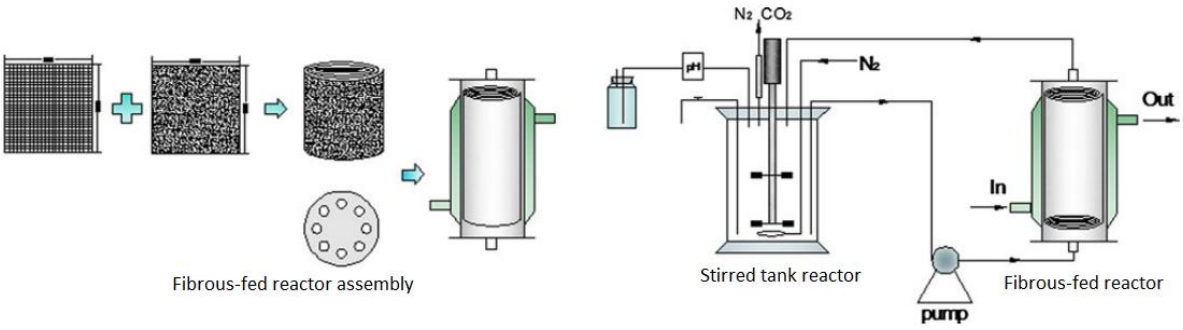

Fig 2. Left side: assembly of the fibrous-fed reactor by packing a spiral wound cotton towel within a jacketed glass column. Right side: stirred tank reactor for pre-culture of P. acidipropionici coupled to the fibrous-fed reactor for immobilization of cells and propionic acid production in a recirculation loop. Figure adapted from [21].

FBB system has also been applied for the fermentation of *Clostridium tyrobutylicum* in order to produce butyric acid, achieving 50 g/L of biomass. Under these conditions, the carbon yield obtained was higher than that accomplished in cell-free fermentation being 50 % for immobilized culture while 35 % for cell-free [22]. Moreover, different other anaerobic microorganisms, including consortia have been investigated under the FBB system. *Clostridium formicoaceticum* and *Lactococcus lactis* were grown together as an immobilized culture in order to produce acetic acid using different complex nitrogen sources and whey, reaching around 30 g/L of biomass. This fermentation was based on the complementary metabolic activity that both microorganisms exhibit; *L. lactis* to produce lactic acid which then is converted to acetic acid by *C. formicoaceticum.* Under this system, the slow-growth microorganism, *C. formicoaceticum,* is first inoculated and immobilized in the wound cotton towel before the production process starts [23].

As aforementioned, cell recycling can be performed by different methodologies like ultrafiltration which can served to reach HDF of anaerobic bacteria as well. Fermentations in which ultrafiltration has been carried out involve the use of *Anaerobiospirillum succiniproducens, Clostridium*

thermoaceticum, Clostridium saccharoperbutylacetonicum among other anaerobic bacteria in cell recycle techniques. This methodology consists in the use of filtration, usually mediated by hollow fiber cartridge-supported system, in order to obtain or "bleed" the biomass and, by using peristaltic pumps, recirculate back the latter into the bioreactor to maintain a controlled levels of cell density in either continuous or sequential batch production[24,25,26]. Figure 3 shows the most basic system in which cell recycling consists, however, adaptions can be performed with the aim to perform a preculture step in a different reactor prior the organic acid production [24]

On the other hand, some microorganisms can be grown-up to HDF levels under aerobic conditions for further anaerobic production process. That is the case of *Corynebacterium glutamicum*, that has been used for the anaerobic production of succinic acid in which the bacteria does not grow under oxygen-scarce conditions but remain metabolically active. As in this case the organic acid production is directly related to the cell density, even the initial production rate, the culture was previously performed aerobically in order to achieve the desired biomass level (40-60 g/L)[27].

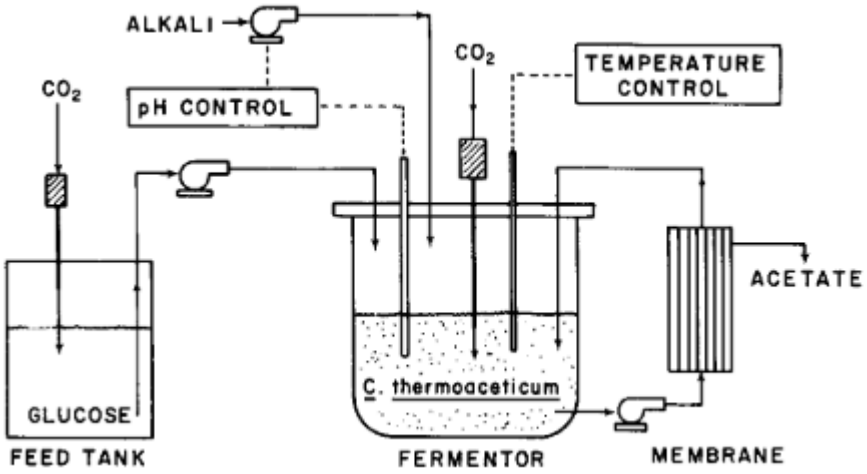

Fig 3. Cell recycle system of a continuous batch process. Feed tank is constantly feeding the fermentor in which the acetate is produced. The culture is transferred by a pump and filtrated by a hollow fiber membrane in order to get the permeate, in which the acetate is, and recirculate the biomass into the fermentor.

5. *Archaea*

Archaea correspond to an organisms' domain that grows under extreme conditions, which confers them the status of extremophiles although not all extremophiles are Archaea as there are species of Eukarya and bacteria that belong to that group as well. These microorganisms possess extremophilic enzymes that allow them to live and reproduce in harsh conditions for other groups of organisms[28,29]. Besides, members of Archaea domain have exhibited a wide range of metabolites, proteins and other kind of compounds that are of biotechnological interest. Despite the high effort

their cultivation requires like high salt concentrations, elevated temperatures or long incubation times, the conditions in which these microorganisms are cultivated present some advantages. Due to the development of the heterologous-expression-of-proteins technology, production at large scale of an enormous number of Archaeal proteins and enzymes have become a reality, especially in mesophilic hosts. This strategy turned out to be very important as it has facilitated the production processes in the biotechnology field as mesophilic bacteria, for instance, *E. coli,* are easier to grow under not very-demanding culture conditions than Archaea. However, these expression systems exhibit several drawbacks. Upon trying to produce biomolecules from a certain microorganism that does not belong to the same domain or even genus of the heterologous host, the machinery for protein folding differs between such different microorganisms, which renders inactive proteins or generation of inclusion bodies. This is due to the contrasting genetic content they possess. In order to overcome these difficulties, a recurrent tool is the modification of the heterologous host at DNA level. However, there are some aspects that cannot be deny when performing mesophilic operations and that is that mesophilic cultures are more susceptible to suffer of infection by non-desired microorganisms. The fact that Archaeon microorganisms require extreme culture conditions such as high temperature to grow or high salt concentrations, results in an advantage when performing wild-type culturing as these conditions are harsh for other microorganisms, preventing external contamination. Moreover, high temperatures in fermentations facilitate the dissolving of compounds which facilitate the substrate intake by microorganisms[30]

HDF is an operation state that can be used to increase the productivity of a biotechnological process based on microorganism culturing. This can be achieved by altering some aspects of the culture conditions, medium composition, supplement addition and fermentation system among others as discussed before. To date, there is no a consensus on what is considered high density fermentation regarding dry weight biomass or optical density but some values have been proposed and they oscillate between 20 g/L and 50 g/L. Establishing specific values for high density fermentation is particularly troublesome as slow-growth microorganism as archaea can barely achieve values around 10 g/L. In these cases, it would be more appropriate to refer to relative HDF, in which biomass values are compared among biotechnological processes carried out for a certain species.

Having said that, *E. coli* is very well known in the production of recombinant proteins, including archaeon protein, by heterologous expression not only for being the most used but also due to the improvements on manufacture of biomolecules apart from biomass. HDF has been accomplished for this model microorganism but there is limited information on high cell density cultures performed on Archaea-domain members as numerous proteins of biotechnological interest that are biosynthesized by these microorganisms have been successfully cloned and produced in heterologous hosts. Microorganisms such as *Pyrococcus furiosus, Pyrococcus abyssi* ST549 *Sulfolobus shibatae, Sulfolobus solfataricus, Methanococcus jannaschii* and some members of the Halobacteriacea family have been considered to be cultured up to high density levels[31,32,28, 33].

As these microorganisms necessitate extreme conditions to grow, suitable adjustments must be performed in fermentation systems such as bioreactor design and operation, and improved media composition for successful HDF operations. Fermentation under thermophilic conditions is one of

the most extensively studied processes along with acidophilic. Thermophilic operations present a major obstacle for microbial growth and that is dissolved oxygen concentration. It is well-known that oxygen exhibit low solubility in aqueous solutions and its solubility decreases as the temperature increase. However, this drawback can be mitigated by the use of stirred-tank bioreactors, especially with the proper selection of impellers that guarantee minimalization of bubble size along with the sparge method which facilitates oxygen transfer from the gas phase to the liquid [28]. On the other hand, fermentations under high concentration of salts need specific adaptations, especially regarding to the material of which bioreactors are made. It is clear that using conventional bioreactors may suffer of corrosion as the high concentration of high electronegative ions such as chloride tend to corrode stainless steel vessels. Bioreactors made of the thermoplastic polyether ether ketone (PEEK) have demonstrated to be very appropriate upon "halothermophilic" bioprocesses as all the stainless-steel connections and compartments are replaced by silicon tubings, PEEK and borosilicate glass. With this system, the archaeon *Haloferax mediterranei* reached values of biomass above 3 g/L using glycerol as substrate, which can be considered HDF (figure 4) [34]. The operational design for this fermentation does not vary much from that used in conventional batch or continuous batch production, as the aspects that change comprises the materials, besides the fact that there is no need of sterilization of medium prior operation due to the high concentration of salts, the probe for dissolved oxygen monitoring must be corrotion-resistant and stirring is done by PEEK-encapsulated magnets [28,35].

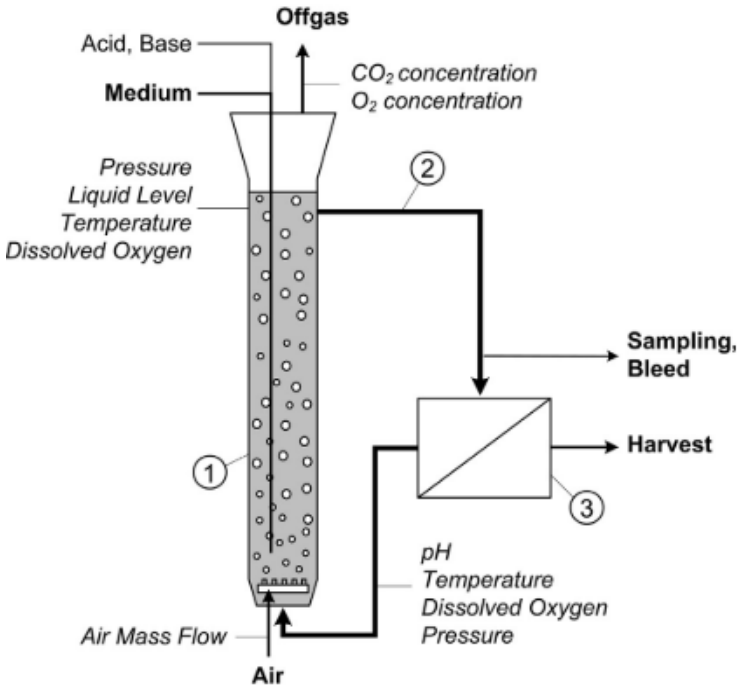

Fig 4. 1 describes the column which is pressure resistant while 2 is designated as the continuous flow to 3 the microfiltration unit performed by pumps.

Similarly to most anaerobes, halophilic archaea present reduced growing rates and their cultivation takes longer than that for aerobic and mesophilic microorganisms and the way to overcome this

drawback also consist in immobilization such as it is performed for bacteria. However, other approaches have been investigated, for instance, the use of membrane bioreactors, rotating biodiscs, sequential batch fermentations and biofilms that allow cell retention and microbial growth of *Halorubrum* sp. in treatment of high-salinity waste water treatment.[36]

Additionally, Some species of archaea have evolved under high-pressure conditions, up to 3 times the atmospheric pressure, in the deep of the oceans. These microorganisms are known as piezophiles and their biotechnological interest relies on the utility of piezoenzymes in food and chemical industry for catalytic reactions in which high pressure is needed. The cultivation of these microorganisms comprises very specific equipped instrumentation and security measures in order to maintain high pressures and the safety for the operational technicians. Unfortunately, no piezophile archaea has been reported to be grown up to HDF to date but a high-pressure system was developed for their cultivation as shown in figure 5. The high cost for the operation of this module limits its application in scaled-up fermentations along with personnel training. Furthermore, in order to be able to meet all the requirements for suitable piezophile growth, the important parameters for their growth must be known beforehand fermentation which is an obstacle as the equipment necessary to collect the data must be specialized and these parameters are closely dependent on the environment and the ecology *in situ* which is troublesome when it comes to simulate the same conditions in laboratories [37].

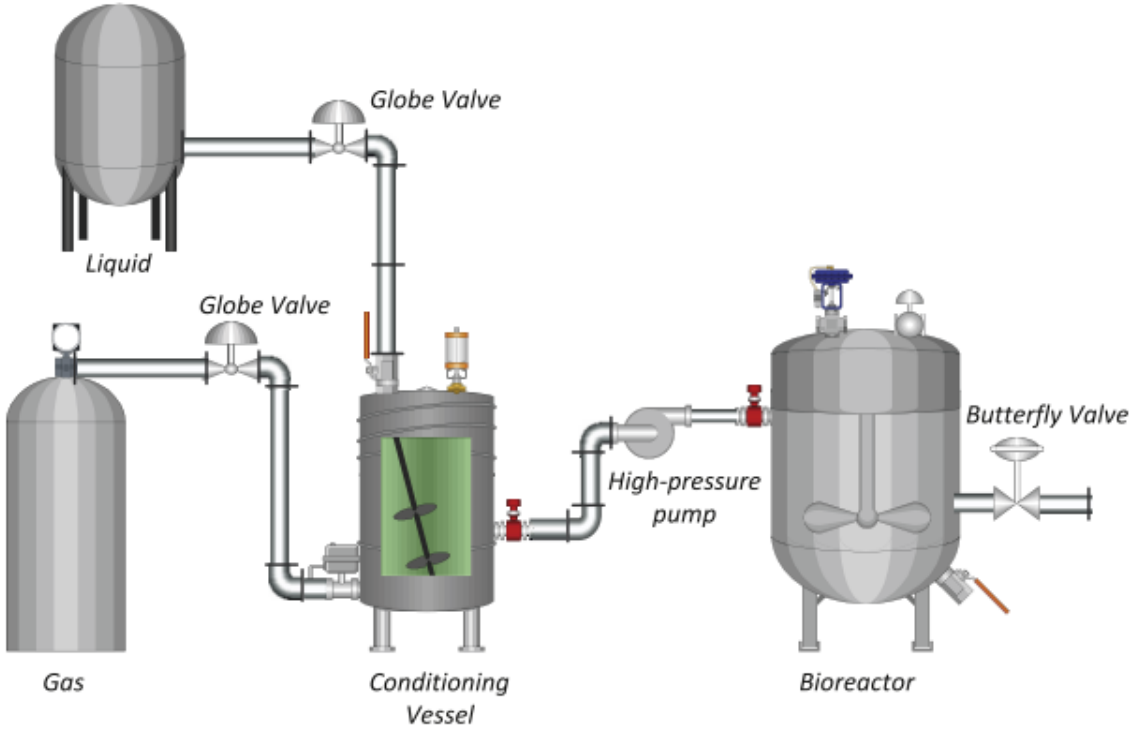

Fig 5. High-pressure fermentation system for batch or continuous operations. Substrate can be supply in the gas inlet which then is dissolved in the conditioning vessel under high pressure for finally transfer the latter fluid to the bioreactor[28,37]

As mentioned before, some species of archaea have been achieved HDF levels during biotechnological operations. One of them is *P. furiosus,* which was grown up to 10^8 cells/mL under a continuous culture regime and using defined minimal media along with starch. Furthermore, *Sulfolobus solfataricus* DSM 1617 reached HDF concentrations when cultured in a fed-batch process with biomass values around 23 g/L when the volume was maintained constant by an evaporated water replacement system while under a microfiltration system as shown in figure 6, 38 g/L of biomass was obtained. In addition, dialysis system has been used in order to prevent product inhibition in *S. shibatae* and *P. furiosus* cultivation, with which 110 g/L and 3 x 10^{-3} cells/mL were achieved respectively, meaning that 20 times more biomass in this fermentation system was obtained compared to batch process [38].

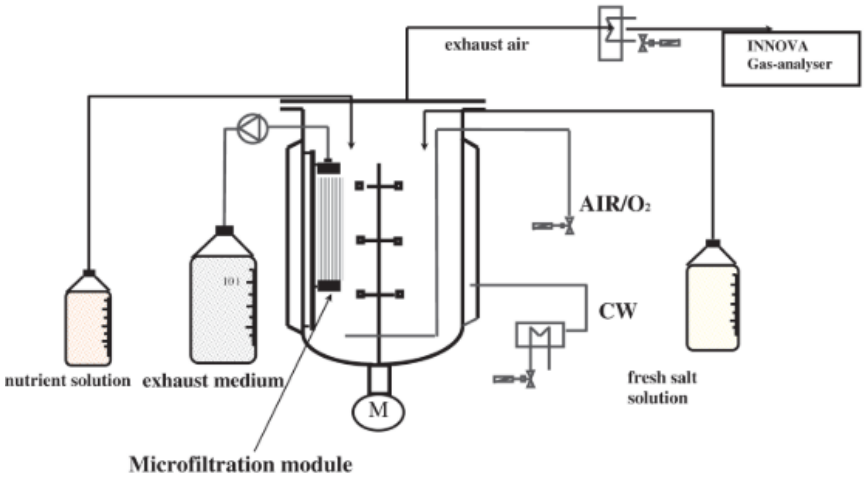

Fig 6. Microfiltration system developed for S. solfataricus in a three-model system in order to prolongate the bacterial growth phase: batch, fed batch and microfiltration.

Another case in archaea HDF is *M. jannaschii* which has been reported to grow up to 2 g/L by improving the nutrient content of medium which was supplemented with Selenium, and increasing the impeller rotational speed up to 600 rpm[38,39]. Several other archeae have achieved HDF values following similar guidelines and fermentation systems already mentioned here and presented in table 1[38]

Microorganism	T_{opt} (°C)	Cultivation mode	Reference
Metallosphera sedula	74	Continuous	Rinker et al. 1999
Methanobacterium thermoautotrophicum	65	Continuous growth-limiting factor H_2	Schills et al. 1996
Methanococcus jannaschii	85	Medium optimization CSTR	Mukhopadhyay et al. 1999
Methanococcus jannaschii	80–85	Continuous	Tsao et al. 1994
Pyrococcus abyssi ST549	95	Continuous	Godfroy et al. 2000
Pyrococcus furiosus	90	Dialysis	Krahe et al. 1996
Pyrococcus furiosus	90	Continuous	Raven et al. 1992
Pyrococcus furiosus	90	Batch on starch-based medium	Raven and Sharp 1997
Pyrococcus furiosus	98	Continuous	Rinker et al. 1999
Sulfolobus shibatae	75	Dialysis	Krahe et al. 1996
Sulfolobus solfataricus Gθ	75	Microfiltration	Schiraldi et al. 1999
Sulfolobus solfataricus MT4	87	Batch on whey-based medium	Romano et al. 1992
Thermococcus barosii	82.5		Duffaud et al. 1998
Thermococcus litoralis	85–88	Batch and continuous	Rinker and Kelly 2000

Tab 1. Archeae species that have been grown up to HDF values and the respective operation regime under this amount of biomass was achieved.

Conclusions

Despite there is a wide variety of operational modules and fermentation systems, there is not general rule that can be applied for reaching HDF values in all the prokaryotes. Every fermentation case must be evaluated in order to establish the most adequate conditions for growth, especially for archaea while the critical parameters in HDF of bacteria are easier to work on and improved. Dialysis fermentation in order to remove toxic contaminants and cell recycle by immobilization are the most attractive approaches for high biomass production in all cases, although different operational modules and bioreactor designs might not allow the system to adjust accordingly for the use of these systems. Despite the great advances in heterologous expression of bio-products, much is yet to be investigated on HDF of archaea in order to avoid drawbacks exhibit by this kind of protein expression.

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