Review Article

AN OVERVIEW OF BIOREACTOR DESIGN FOR PROTEIN AND ENZYMES ENRICHEMENT OF AGRICULTURAL RESIDUES BY SOLID STATE FERMENTATION

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ABSTRACT

In this review article we discuses about bioreactor designs and their use for protein production under solid state fermentation (SSF) conditions using various agricultural by-products. The advantages and disadvantages of various bioreactors and their potential for scale-up are described. SSF is proposed as a suitable low-tech strategy for protein enrichment for animal feed by converting a previously low value substance into a more nutritionally valuable one. The use of various substrates and microorganisms for protein enrichment are also listed. Solid-state fermentation has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products. Its application in bioprocesses such as bioleaching, bio-beneficiation, bio-remediation, bio-pulping, etc. has offered several advantages. Utilization of agro-industrial residues as substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilized residues. Today with better understanding of biochemical engineering aspects, particularly on mathematical modeling and design of bioreactors (fermenters), it is possible to scale up SSF processes and some designs have been developed for commercialization. It is hoped that with continuity in current trends, SSF technology would be well developed at par with submerged fermentation technology in times to come.

INTRODUCTION

Solid state fermentation (SSF) may be characterized by a fermentation process carried out on a solid medium with a low moisture content *(Aw)*, typically 0.40-0.90, which occurs in a non-septic and natural state. SSF has been successfully exploited for food production, fuel, enzymes, animal feeds and also for dye degradation. Many of the solids used for SSF are unrefined and are of agricultural origin making complete characterization and exact reproducibility difficult. In recent years, SSF has received more and more interest from researchers, as studies have demonstrated superior product yields and simplified downstream processing. The use of solid matter, either as an inert support or substrate/support has, however, had serious implications on the engineering aspect of bioreactor design and operation. The low moisture content means that fermentation can only be carried out by a limited number of microorganisms, mainly yeasts and fungi, although some bacteria have been used. This means that although SSF is a non-septic fermentation, spoilage or contamination by unwanted bacteria is reduced by the low *Aw* (<0.95), inhibits most bacterial growth. Therefore, as a result of the low *Aw* in SSF bioreactors, smaller fen enters is required and a more concentrated product is produced, simultaneously reducing energy requirements for downstream processing. Sterilization costs are lower due to the limitation of free water, which in turn reduces operating costs needed for effluent treatment.

Depending upon the nature of the substrate, the amount of water absorbed could be one or several times more than its dry weight, which leads relatively high water activity (a_w) on the solid/gas interface in order to allow higher rate of biochemical process. Low diffusion of nutrients and metabolites takes place in lower water activity conditions whereas compaction of substrate occurs at higher water activity. Hence, maintenance of adequate moisture level in the solid matrix along with suitable water activity is essential elements for SSF processes. Solid substrates should have generally large surface area per unit volume (in the range of 10^3-10^6 m^2/cm^3 for the ready growth on the solid/gas interface). Smaller substrate particles provide larger surface area for microbial attack but pose difficulty in aeration/respiration due to limitation in inter-particle space availability. Larger particles provide better aeration/respiration opportunities but provide lesser surface area. In bioprocess optimization, sometimes it may be necessary to use a compromised size of particles (usually a mixed range) for the reason of cost effectiveness. For example, wheat bran, which is the most commonly used substrate in SSF, is obtained in two forms, fine and coarse. Former contains particles of smaller size (mostly smaller than 500-600μ). Most of SSF processes use a mix of these two forms at different ratios for optimal production. Solid substrates generally provide a good dwelling environment to the microbial flora comprising bacteria, yeast and fungi. Among these, filamentous fungi are the best studied for SSF due to their hyphal growth, which have the capability to not only grow on the surface of the substrate particles but also penetrate through them. Several agro crops such as cassava, barley, etc. and agro-industrial residues such as wheat bran, rice bran, sugarcane bagasse, cassava bagasse, various oil cakes (e.g. coconut oil cake, palm kernel cake, soybean cake, ground nut oil cake, etc), fruit pulps (e.g. apple pomace), corn cobs, saw dust, seeds (e.g. tamarind, jack fruit), coffee husk and coffee pulp, tea waste, spent brewing grains, etc are the most often and commonly used substrates for SSF processes. During the growth on such substrates hydrolytic exo-enzymes are synthesized by the

micro-organisms and excreted outside the cells, which create and help in accessing simple products (carbon source and nutrients) by the cells. This in turn promotes biosynthesis and microbial activities.

A large amount of metabolic heat is generated during SSF and its rate is directly proportional to the level of metabolic activity in the system. Heat transfer in SSF reactors is not as efficient in comparison to submerged (liquid) fermentations (SmF). This is mainly due to the solidness of the substrate and the lack of free water available during fermentation. In laboratory scale reactors, heat may be removed by keeping the culture vessel in a temperature-controlled environment, such as a water bath. Problems in SSF occur when scale-up is considered; the problem of the lack of free water and generation of metabolic heat is greatly exaggerated as the system struggles to provide adequate agitation, aeration and cooling. Temperatures have been reported to be as high as 47-50°C on the centre of the fermenting mass by Rat bun and Shuler and temperatures as high as 60-70°C by Hayes in the innermost region. The use of SSF for protein enrichment of lignocelluloses residues has received close attention due its low level technology, reduced reactor volume per unit weight of substrate converted and its direct applicability of the fermented product for feeding purposes. Large quantities of fibrous crop residues are currently under utilized as potential animal feed sources, especially in developing countries. A major reason for this is the low protein content of the waste residues, which can be enriched utilizing added urea as nitrogen source through fermentation by white-rot fungi.

SOLID STATE FERMENTATION (SSF):

Solid-state (substrate) fermentation (SSF) has been defined as the fermentation process occurring in the absence or near-absence of free water. SSF processes generally employ a natural raw material as carbon and energy source. SSF can also employ an inert material as solid matrix, which requires supplementing a nutrient solution containing necessary nutrients as well as a carbon source. SSF has been considered superior in several aspects to submerged fermentation [SmF] due to various advantages it renders. It is cost effective due to the use of simple growth and production media comprising agro-industrial residues, uses little amount of water, which consequently releases negligible or considerably less quantity of effluent, thus reducing pollution concerns. SSF processes are simple, use low volume equipment (lower cost), and are yet effective by providing high product (concentrated products). Further, aeration process (availability of atmospheric oxygen to the substrate) is easier since oxygen limitation does not occur as there is an increased diffusion rate of oxygen into moistened solid substrate, supporting the growth of aerial mycelium. These could be effectively used at smaller levels also, which makes them suitable for rural areas also.

GENERAL ASPECT OF SSF:

There are several important aspects, which should be considered in general for the development of any bioprocess in SSF. These include selection of suitable microorganism and substrate, optimization of process parameters and isolation and purification of the product. Going by theoretical classification based on water activity, only fungi and yeast were termed as suitable micro-organisms for SSF. It was thought that due to high water activity requirement, bacterial cultures might not be suitable for SSF. However, experience has shown that bacterial cultures can be well managed and manipulated for SSF processes. It has been generally claimed that product yields are mostly higher in SSF in comparison to SmF. However, so far there is not any established scale or method to compare product yields in SSF and SmF in true terms. The exact reasoning for higher product titers in SSF is not well known currently. The logical reasoning given is that in SSF microbial cultures are closer to their natural habitat and probably hence their activity is increased.

Selection of a proper substrate is another key aspect of SSF. In SSF, solid material is non-soluble that acts both as physical support and source of nutrients. Solid material could be a naturally occurring solid substrate such as agricultural crops, agro-industrial residues or inert support. However, it is not necessary to combine the role of support and substrate but rather reproduce the conditions of low water activity and high oxygen transference by using a nutritionally inert material soaked with a nutrient solution. In relation to selection of substrate, there could be two major considerations; one that there is a specific substrate, which requires suitable value-addition and/or disposal. The second could be related with the goal of producing a specific product from a suitable substrate. In the latter case, it would be necessary to screen various substrates and select the most suitable one. Similarly it would be important to screen suitable micro-organisms and select the most suitable one. If inert materials such as polyurethane foam are used, product isolation could be relatively simpler and cheaper than using naturally occurring raw materials such as wheat bran because while extracting the product after fermentation, along with the product, several other water-soluble components from the substrate also leach out and may pose difficulties in purification process. Inert materials have been often used for studying modeling or other fundamental aspects of SSF.

BIOCHEMICAL ENGINEERING ASPECTS OF SSF:

In recent years, several excellent reports have appeared providing a great deal of knowledge and understanding of the fundamental aspects of SSF and today we know much better information about the heat and mass transfer effects in SSF processes, which have been considered as the main difficulties in handling SSF systems. However, there still remains much to be done in this regard. During SSF, a large amount of heat is generated, which is directly proportional to the metabolic activities of the micro-organism. The solid materials/matrices used for SSF have low thermal conductivities; hence heat removal from the process could be very slow. Sometimes accumulation of heat is high, which denatures the product formed and accumulated in the bed. Temperature in some locations of the bed could be 20°C higher than the incubation temperature. In the early phases of SSF, temperature and concentration of oxygen remain uniform throughout the substrate but as the fermentation progresses, oxygen transfer takes place resulting in the generation of heat. The transfer of heat into or out of SSF system is closely related with the aeration of fermentation system. The temperature of the substrate is also very critical in SSF as it ultimately affects the growth of the micro-organism, spore formation and germination, and product formation. High moistures results in decreased substrate porosity, which in turn prevents oxygen penetration. This may help bacterial contamination. On the other hand, low moisture content may lead to poor accessibility of nutrients resulting in poor microbial growth.

ADVANTAGES AND DISADVANTAGES OF SSF:

ADVANTAGES:

- The culture media are simple. Some substrates can be used directly as a solid media or enriched with nutrients.
- The product of interest is concentrated, that which facilitates its purification.
- The used inoculums is the natural flora of the substrates, spores or cells.
- The low humidity content and the great inoculums used in a SSF reduce vastly the possibility of a microbial contamination.
- The enzymes are low sensitive to catabolic repression or induction.
- The quantity of waste generated is smaller than the SmF

For the development of bioprocess such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop-residues for nutritional enrichment, biopulping, and production of value-added products such as biologically active secondary metabolites, including antibiotics, alkaloids, plant growth factors, enzymes, organic acids, biopesticides, including mycopesticides and bioherbicides, biosurfactants, biofuel, aroma compounds, etc.

DISADVANTAGES:

- The used microorganisms are limited those that grow in reduced levels of humidity.
- The determination of parameters such as humidity, pH, free oxygen and dioxide of carbon, constitute a problem due to the lack of monitoring devices.
- The scale up of SSF processes has been little studied and it presents several problems.

BIOREACTOR:

A bioreactor is a vessel in which is carried out a chemical process which involves organisms or biochemically active substances derived from such organisms. Bioreactors are commonly cylindrical, ranging in size from some liter to cube meters, and are often made of stainless steel. Bioreactor design is quite a complex engineering task. Under optimum conditions the microorganisms or cells will reproduce at an astounding rate. The vessel's environmental conditions like gas (i.e., air, oxygen, nitrogen, carbon dioxide) flow rates, temperature, pH and dissolved oxygen levels, and agitation speed need to be closely monitored and controlled.

DESIGN OF BIOREACTOR FOR SSF:

Over the last decade, there has been a significant improvement in understanding of how to design, operate and scale up SSF bioreactors. The key to these advances has been the application of mathematical modeling techniques to describe various physicochemical and biochemical phenomena within the system. The basic principle of SSF is the "solid substrate bed". This bed contains the moist solids and an inter particle voids phase. SSF has been conventionally more applicable for filamentous fungi, which grow on the surface of the particle and penetrate through the inter particle spaces into the depth of the bed. The process in most of the cases is aerobic in nature. The suitable bioreactor design to overcome the heat and mass transfer effects, and easy diffusion and extraction of metabolites has become the topic of hot pursuit. While tray and drum type fermenters have been studied and used since long, much focus has been paid in last few years on developing packed bed fermenters as they could provide better process economics and a great deal of handling ease . A tray bioreactor could have unmixed beds without forced aeration of (manually) mixed bed without forced aeration. However, there have been no significant advances in tray design. Packed beds could be unmixed beds with forced aeration and rotating drums could have intermittent agitation without forced aeration, operating on continuous or semi-

continuous mode. The bed could be agitated intermittently or continuously with forced aeration.

CURRENT SSF STRATEGIES FOR SCALE UP OF BIOREACORS:

Many of the current research papers concerning SSF have tended to focus on reporting lab-scale findings. This is understandable, as parameters tend to be more controllable, with data collection simplified and running costs low. There are therefore few papers describing current state of the art reactors, giving design and yield details, as this is industrially sensitive material. Fermentation strategies have scaled-up and adopted to suit various situations and needs. Singh and Gupta, describe the SSF of cereal straw under a polythene cover, known as the "Karnal process". This method is carried out in two stages, and uses thinly layered substrate as in a tray fermenter, but without the use of a perforated tray support. In the first stage, the cereal straw is treated with 4% urea at a 40% moisture level and is ensiled under the polythene cover for 30 days. The second stage involves the use of a rectangular brick structure (200 cm x 150 cm) which acts as the bioreactor. The loosely stacked bricks help provide aeration from all sides, with a thin layer of urea-treated straw spread thinly inside the brick enclosure as a nitrogen source for the fungi. *Coprinus fimetarius* was added and its subsequent growth enriched the protein content of the straw. Although external parameters could not be finely controlled, the brick reactor and the covering of polythene helped to maintain an adequate balance between heat generation and loss, and also allow a reasonable level of aeration.

MATERIALS AND METHOD:

Solid State Fermentation (SSF): Term solid state fermentation (SSF) is applied for the processes in which insoluble materials in water are used for the microbial growth. In the fermentative processes of this type, the quantity of water should not exceed the capacity of saturation of the solid bed in which the microorganisms growth. Water is essential for the microbial growth and in SSF and it is present in thin layers and in occasions, absorbed inside the substrates.

In the western world the SSF has been fewer studied that the SmF and SLF. The most important differences among these systems are the relatively low content of humidity in the means, the formation of gradients of temperature, nutrients and products andalso, sporulation mechanisms as well as the production of enzymes and secondary metabolites as the rifamycin, citric acid and aromas. Substrates from agricultural or industrial wastes (wheat straw or barley, sugar cane bagasse, coffee pulp, grape wastes, copra pasta, among other) or inert materials (as resins of ionic exchange, acreolite or poliurethane foam) can be used. The pretreatments of these materials is really few, generally a milled previous and wash. Of these characteristics some advantages and disadvantages of the SSF in comparison to the SmF are derived and presented next.

SSF SUBSTRATES FOR PROTIEN ENRICHEMENT BY SSF:

As previously mentioned crop residues represent a potential source of dietary energy to ruminants if the protein content of can be enriched. As these residues are renewable and in an abundant supply (~3.5 billion tones of agricultural by-products per year) they represent a potential.

Table 1: Various substrates and microorganisms used for protein enrichment of agricultural waste residues (some examples):

Substrate	Microorganism
Apple pomace	*C.utilis, Kloeckera apiculata, S. cerevisae, A. Niger, C. tropicalis,Trichoderma viriae*
Grape stalks, orange peels	*Agrocybe aegirata, Amillarialla mellea, P. ostreatus*
Carob pods	*A. Niger*
Sugarcane baggase	*Chaetomium celluloticum*

SSF of lignocelluloses material:

Lignocelluloses crop residues may be characterized by being high in cellulose, hemicelluloses and lignin, but low in protein. They tend to be difficult to digest by ruminants and so are limited as potential animal feed. The SSF of these residues has been explored using fungi for protein enrichment. The use of temperate lignocelluloses wastes, such as wheat straw, has been examined by many researchers. The use of rice and maize straw in developing countries may also be exploited.

SSF of citrus wastes:

Citrus wastes are wastes remaining after juicing on an industrial scale. These dried peels contain simple sugars, pectin and cellulose but are low in protein. A. Niger has been reported to utilize the dried citrus peels under SSF conditions, by fermenting the simple sugars present.

SSF of apple pomace:

After pulping, apple pomace, which consists of crushed skins, pips and stalks, may be fermented for protein enrichment. It has high sugar content, as well as a high moisture content (~80%) which poses disposal problems for the pulping industry. Unfermented apple pomace had been previously fed directly to pigs, but was mostly dumped in landfill sites. The use of co-cultures of yeasts and fungi to enrich the protein content of the pomace. Protein and pectin increased by 20 and 17%, respectively, when apple pomace was fermented by Candida utilis and A. Niger under SSF conditions.

SSF of carob pobs:

Carob or locust tree (Ceratunia siliqua) is found extensively in Mediterranean countries, with the pods containing large amounts of sugars, making them an

attractive substrate for protein enrichment by SSF. Carob pods also contain high amounts of tannin, which has an adverse effect on animal growth. Tannins may be degraded by certain fungi, including A. Niger whilst also being simultaneously enriching the protein content of the carob pods. Protein enrichment of 20% was achieved in 4 days of SSF with an 83% tannin decrease.

PRODUCTION OF ENZYMES BY SSF:

In studies where the enzyme concentration obtained from SmF, SLF and SSF have been compared, it has been observed that in SSF the titles are higher and also, the produced enzymes are stable to wider ranges of temperature and pH. This has been evidenced for enzymes such as the amylase, pectinase, tannase and protease. In these studies it has demonstrated that SSF presents attractive advantages for the production of microbial enzymes. More recent studies in SSF involve the production of enzymes with potential utilization in the food industry. Such enzymes are a) tannin acyl hydrolase, b) protopectinase, c) protease, on those which next some of the most important characteristics are revised, including the reaction that catalyze and the possible industrial applications.

Tannase:

Tannase or tanin acyl hydrolase catalyzes the hydrolysis reaction of the ester bonds present in the hydrolizable tannins and gallic acid esters. Its industrial production is only by a microbial way using SmF, where the enzymes are mainly produced intracellularly, implying additional costs in its manufacture. Recently, tannase is commercialized, with different catalytic units depending of the product presentation. However several studies have reported interesting advantages between the tannase produced by SSF in comparison with that produced by SmF. In these works, attractive advantages are indicated, such as the high enzymatic production (up to 5.5 times more than in SmF), the nature extracellular of the enzymes and the stability to wide pH and temperature ranges reported a tannase productivity of 6.667 and 1.275 UE/Lh for SSF and SmF respectively and a maximum of intracellular and extracellular tannase activity respectively 18 and 2.5 times higher in SSF that in SmF. At the moment, the biggest commercial applications of tannases are in the manufacture of instantaneous tea or of acorn liquor and in the gallic acid production which is used as an important intermediary compound in the food and pharmaceutical industry, respectively for the synthesis of propylgallate and trimethoprim. Also, the tannase is used as clarifying agent in some wines, juices of fruits and in refreshing drinks with a coffee flavor. Fifty percent of the wine color is due to the presence of tannins; however, if these compounds are oxidized to quinones by contact with the air, an undesirable turbidity could be formed, affecting the wine quality. The use of tannases can solve this problem. Tannins are present in low quantities in beer, especially as anthocianins. However, when beer proteins are present in high quantities, an undesirable turbidity is presented by complexing with these tannins. This problem could be solved with the employment of tannases. The tannery effluents contain high amounts of tannins, mainly polyphenols, which are dangerous pollutants, for this reason the use of the tannase represents an inexpensive and effective treatment forth removal of these compounds.

Pectinases:

Most of pectic-polymers are comprised of smooth homo-galacturonan and ramified hairy regions. Smooth regions consist of a linear homo-galacturonan backbone, while hairy regions consist of rhamnogalacturonan backbone with side-branches of varying length. Detailed information on elaborate models on pectin structure can be found in some reviews. Traditional breaking down of pectic substances was through the use of well-know pectic enzymes (pectinesterase, poly-galacturonase and pectin lyase) able to degrade only the smooth region. Recently, several new enzymes have been reported.

Proteases:

Proteases have an important position in the enzyme industry as they have a determinant role in the microbial and human physiological needs as well as the great commercial market applications. Since they are indispensable for living organisms, proteases occur in a wide diversity of plants, animals and microorganisms. Papain, bromelain, keratinases and ficsin represent some of the plant proteases; however, the use of plants as a source of proteases is governed by factors no easily controlled such as land availability and climatic conditions as well as its excreta ion is a time-consuming process. Pancreatic, trypsin, chymotrypsin, pepsin and rennin are the most important proteases of animal origin. However, their production depends on the availability of livestock for slaughter. Therefore microbial proteases are preferred above enzymes from plant and animal sources since they present most of the desired characteristics for biotechnological applications.

CONCLUSION

The potential advantages of SSF for protein enrichment can be seen if scale-up parameters, such as cooling and heat transfer can be more easily controlled. The low *Aw*, although benefiting the inoculums in terms of competition, has a detrimental effect on the basis of heat transfer. Metabolic heat generated by rapidly growing microorganisms due to better mixing and aeration of reactors, also poses problems for the SSF system affecting product formation. Additional heat through the friction of mixing supplements to the problems of heat

exchange. SSF can produce a more concentrated protein product that may be used as an animal feed in both developing and developed countries. As SSF bioreactors have increased in size in order to try and increase the product concentration so too have the problems concerning parameter controls. The benefits of SSF for protein enrichment maybe better realized in situ, on farms in developing countries, which can avail of this relatively low-tech fermentation system. It is apparent that SSF may be a viable technology for the enrichment of previously worthless waste residues for animal feed.

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