Chapter 15

Production of Microbial Biosurfactants by Solid-State Cultivation

Nadia Krieger,* Doumit Camilios Neto and David Alexander Mitchell

Abstract

In recent years biosurfactants have attracted attention because of their low toxicity, biodegradability and ecological acceptability. However, their use is currently extremely limited due to their high cost in relation to that of chemical surfactants. Solid-state cultivation represents an alternative technology for biosurfactant production that can bring two important advantages: firstly, it allows the use of inexpensive substrates and, secondly, it avoids the problem of foaming that complicates submerged cultivation processes for biosurfactant production. In this chapter we show that, despite its potential, to date relatively little attention has been given to solid-state cultivation for biosurfactant production. We also note that this cultivation technique brings its own challenges, such as the selection of a bioreactor type that will allow adequate heat removal, of substrates with appropriate physico-chemical properties and of methods for monitoring of the cultivation process and recovering the biosurfactants from the fermented solid. With suitable efforts in research, solid-state cultivation can be used for large-scale production of biosurfactants.

Introduction

Surfactants are amphipathic molecules that reduce the surface tension at oil-water or air-water interfaces. They have applications in many areas, including environmental protection, petroleum production and cosmetics. The great majority of surfactants used in these applications and available on the market are produced by chemical synthesis routes. There is currently interest in replacing these chemical surfactants with surfactants of biological origin, the so-called "biosurfactants", which usually are of lower toxicity and more easily biodegradable.[1-5]

Biosurfactants have their best potential market in applications in which it is necessary to disperse tensioactive agents in the environment, for example, in the cleaning of spills of oils and other hydrophobic compounds and in the enhancement of recovery of oil from reservoirs. Beyond this, they can be used to improve the quality of oil, in the synthesis of new polymers, as additives to cosmetics and in the synthesis of bioplastics.[6-8] However, despite their potential, the use of biosurfactants in these applications is currently extremely limited, the major reason being that the cost of production of biosurfactants is very high in relation to the cost of production of chemical surfactants. As a result, there are no commercial large-scale processes for biosurfactant production.[9] It will be necessary to reduce production costs significantly before biosurfactants can find widespread use.

Most research into the production of biosurfactants has been undertaken using submerged cultivation of the producing microorganism. However, this production method creates serious problems with foam formation. Solid-state cultivation is an alternative method for the production of microbiological products that has the potential to avoid these problems. However, there has

*Corresponding Author: Nadia Krieger—Chemistry Department, Federal University of Parana, PO Box 19081, Curitiba 81531-990, PR Brazil. Email: nkrieger@ufpr.br

Biosurfactants, edited by Ramkrishna Sen.

been relatively little research into the production of biosurfactants by solid-state cultivation and, further, this cultivation technique has its own challenges.

In this chapter we explore the potential advantages that solid-state cultivation technology can bring to the production of biosurfactants. We show that a relatively small amount of work has been done in this area and outline future investigations that will need to be undertaken.

Microbial Biosurfactants That It Would Be Interesting to Produce at Large Scale

A variety of microorganisms can produce biosurfactants. Biosurfactants are generally produced as a mixture of compounds of the same chemical group ("congeners" or "chemical homologues"). The composition of the mixture depends on the strain of microorganism and on the conditions under which it is cultivated and may affect the physicochemical properties of the biosurfactant.

According to Zajic and Seffens,[10] biosurfactants can be classified in five major groups: (1) glycolipids; (2) lipopolysaccharides; (3) lipopeptides; (4) phospholipids; (5) fatty acids and neutral lipids. Each group of biosurfactants presents distinct physicochemical properties and specific physiological functions, the majority being constituted by different hydrophilic and hydrophobic moieties. Most biosurfactants are anionic or neutral, but some are cationic, as is the case of those that contain amino-groups. The hydrophobic moiety can be formed by long-chain fatty acids, hydroxy-fatty acids or by α-alkyl-β-hydroxy-fatty acids, while the hydrophilic portion of the molecule is composed of carbohydrates, amino acids, cyclic peptides, phosphate, carboxylic acids or alcohols.[11]

To date most interest in developing processes for the production of microbial biosurfactants has focused on glycolipids and lipopeptides. In the case of glycolipids, there have been studies into the production of rhamnolipids by *Pseudomonas aeruginosa*, of sophorolipids by *Candida bombicola* and of mannosylerythritol lipids by *Pseudozyma* (previously *Candida*) *antarctica*.[6] In the case of lipopeptides, there has been interest in the use of strains of *Bacillus* to produce molecules like surfactin, iturin and fengycin.[6] Since these biosurfactants (at least the general family types) have been covered in other chapters of this book, we will not discuss their properties in any detail. The important point, which will be relevant in terms of the solid-state cultivation production technology, is that they are produced by unicellular, aerobic organisms.

Production of Biosurfactants by Classical Submerged Cultivation Is Problematic

The great majority of studies into the production of biosurfactants uses submerged cultivation. This is also the case in the few small scale production processes that exist, such as the production of the lipopeptide biosurfactant of *Bacillus subtilis,* Surfactin (Sigma-Aldrich Co.).

The microorganisms typically used for the production of biosurfactants are aerobic organisms. Therefore submerged cultivation processes are conducted with forced aeration and agitation. However, this creates a serious problem when the biosurfactant starts to be produced because large quantities of foam are produced.[12-14] This foaming has several deleterious effects. Firstly, there is a tendency for the microorganism to accumulate within the foam, thereby removing cells from the culture medium.[13,14] Secondly, the presence of the foam reduces the efficiency of gas transfer between the gas and liquid phases in the bioreactor, reducing the rates of supply of oxygen to the liquid and removal of carbon dioxide from it. Thirdly, the foaming is typically so severe that the foam tends to leave the headspace through any available orifice. This not only represents a loss of cells from the system but also greatly increases the risk of contamination of the bioreactor.

Two main strategies have been used to combat the problem of foaming during the production of biosurfactants in submerged cultivation: the addition of antifoaming agents and mechanical breakage of the foam. However, neither solution is particulary attractive. The addition of antifoaming agents brings three disadvantages:[13,14] Firstly, the most efficient antifoaming agents are organic mixtures based on polypropylene or polymers derived from silicone and these are relatively expensive. Secondly, antifoaming agents decrease the efficiency of oxygen and carbon dioxide

transfer between the gas and liquid phases and may even be toxic to the microorganism. Thirdly, antifoaming agents represent a "chemical contaminant" that must be later separated from the biosurfactant during downstream processing.

Mechanical devices for breaking the foam may be internal or external to the bioreactor. Internal devices include foam-breakers in the headspace of the bioreactor (typically mounted on the agitator shaft). However, such foam breakers are not effective when large quantities of foam are produced, as is the case in biosurfactant production processes. It is therefore necessary to install external devices in which the foam is collapsed. The cells and medium removed by the foam can then be recycled back to the bioreactor. However, such systems must operate aseptically and make the construction and operation of the bioreactor significantly more expensive.[13,15,16]

Solid-State Cultivation as an Alternative Cultivation Technique with Potential for Biosurfactant Production

In the face of the problems with biosurfactant production in submerged cultivation, solid-state cultivation is an interesting alternative.

Solid-state cultivation involves the growth of microorganisms on moist organic solid particles, within beds in which there is a continuous gas phase between the particles.[17] The majority of water in the system is absorbed within the solid particles. There is relatively little free liquid water in the interparticle spaces, being limited to a thin film on the surface of the particles and possibly a few small droplets. This "architecture" of the system in solid-state cultivation avoids the foaming problem that plagues submerged cultivation processes for biosurfactant production. Even though in some bioreactors air is blown forcefully through the bed, since the air passes through the interparticle spaces and is not sparged through a liquid containing biosurfactant, foam does not form in the first place.

The difference in system architecture also has an important consequence for the design of large-scale bioreactors. Typically the major consideration in the design of bioreactors for aerobic submerged cultivation processes is the maintenance of sufficiently high rates of gas-to-liquid mass transfer, in order to maintain an acceptably high dissolved oxygen concentration. In the case of solid-state cultivation processes the major consideration is the maintenance of sufficiently high rates of heat removal, in order to maintain the temperature of the substrate bed as close as possible to the optimal temperature for growth and product formation. However, in other respects, solid-state cultivation processes are similar to submerged cultivation processes. In other words, there is a need for upstream processes, including the production of a suitable inoculum and substrate preparation, and downstream processes, including product recovery, purification and waste disposal.

What Is the State of the Art of Biosurfactant Production in Solid-State Cultivation?

Despite the potential advantages that solid-state cultivation has for the production of biosurfactants, there has been relatively little effort to develop processes. The earliest work was done by Ohno et al.[18-22] In fact, their aim was not actually to produce biosurfactant, but rather to produce compounds with antibiotic activity against phytopathogens: They isolated strains of *Bacillus subtilis* that produced the cyclic lipopeptides iturin A and surfactin, these cyclic peptides having both antibiotic activity and surfactant properties. The majority of their studies were undertaken in Erlenmeyer flasks, although they did undertake one study in which in which 3 kg of *okara* (a residue of the manufacture of tofu) was placed in an 8-litre jar. This jar was placed in a waterbath and "air was supplied through silicon-rubber tubing connected to a compressor".[22] No more detail is presented than this, so it is not possible to determine whether the bed would have been aerated effectively. In fact it is not even made clear whether the tubing was placed in such a manner that the air was forced to cross the bed in order to leave the jar. In any case, temperature control in this "jar bioreactor" was very poor: despite the fact that the waterbath was maintained at 23°C, the temperature within the jar rose to 45°C. In fact, it should be noted that *okara* does not have properties that suit it well to the realization of

large-scale solid-state cultivation processes in bioreactors. It has quite a small particle size and a tendency to form a paste at high water contents.

Later, Veenadig et al[12] studied the production of surfactants by *Bacillus subtilis* cultivated on wheat bran. In these studies they did not identify the particular biosurfactant produced, nor did they use specific analytical methods like HPLC. Rather, they analyzed the performance of their cultivations in terms of the emulsifying activity and the reduction in surface tension provoked by samples removed during the cultivation. The packed bed was a stainless steel column of 15 cm diameter and 34.5 cm height. Samples from the packed-bed were removed from a sampling orifice at 17 cm bed height. Note that since gradients are typical of packed-bed bioreactors, these samples did not give a clear picture about what was happening in the bioreactor as a whole. In one study they investigated the effect of the aeration rate used during the cultivation, over the range of 10 to 20 L/min. The surface tension measured when samples removed from the bed were added to water was lower for the higher air flow rates. In other words, the samples corresponding to the higher air flow rates provoked greater reductions in surface tension. Pure water has a surface tension of 72 dynes/cm. Samples removed at the beginning of the cultivations reduced the surface tension to around 55 dynes/cm. For the aeration rate of 10 L/min, the samples removed during the cultivation were not able to reduce the surface tension to values below 50 dynes/cm. On the other hand, a sample removed at 55 h from the packed bed operated with an aeration rate of 20 L/min was able to reduce the surface tension to a value as low as 24 dynes/cm. Veenadig et al[12] also evaluated the performance of flask cultivations. Over the first 30 h the surface tensions obtained with samples removed from the flasks were quite similar to these obtained in the packed bed with an aeration rate at 20 L/min (the surface tension measured when the samples were added to water fell from an initial value of 55 dynes/cm to values of around 35 dynes/cm for the 30 h sample). Longer cultivation times did not lead to lower surface tensions.

More recently, Das and Mukherjee[23] studied the production of lipopeptide biosurfactants by two thermophilic strains of *Bacillus subtilis*. The substrate used was waste potato peels, which were washed, blanched (80°C), dried, ground, redried and then autoclaved. Both submerged cultivation and solid-state cultivation were studied; in both cases the experiments were done in Erlenmeyer flasks. In the case of submerged cultivation the potato peel was added at a concentration of 2% (m/v) to a mineral salt medium. In the case of solid-state cultivation 2 mL of this mineral salt medium was added per 5 g of ground potato peels. The comparison between submerged and solid-state cultivation was undertaken on the basis of the amount of biosurfactant produced per gram of dry solids. On this basis the levels of biosurfactant produced were reasonably similar in the two systems, for the better-producing strain being 80 mg per gram of dry solids for submerged cultivation and 67 mg per gram of dry solids for solid-state cultivation. These values were increased to 102 and 92 mg per gram of dry solids, respectively, when glucose was added to the mineral salts medium (at a level of 0.5% m/v).

In our own work we have produced rhamnolipids by *Pseudomonas aeruginosa* in solid-state cultivation.[24] In this case sugar cane bagasse was used as a support material and was impregnated with a solution containing mineral salts and glycerol. Cultures were undertaken in Erlenmeyer flasks. Rhamnolipids were extracted from the fermented solid and quantified in terms of the amount of rhamnose produced. The performance of the solid-state cultivation was compared with that of a submerged culture done in Erlenmeyer flasks (note that the bacterium was also cultivated within a bioreactor but, as soon as rhamnolipid production started, the foaming problem was so severe that all the liquid was lost from the bioreactor in a short space of time). The comparison was done on the basis of the amount of rhamnolipids produced per volume of nutrient solution. In the case of submerged culture, this was the total volume of nutrient solution in the flask. In the case of solid-state cultivation, this was the amount of nutrient solution added to the sugar cane bagasse. Production was similar in both systems, reaching 1.6 g of rhamnose per litre at 144 h, corresponding to a level of 8.0 g per kg of dry fermented substrate.[25] We are undertaking further studies with the aim of improving the productivity of the solid-state cultivation system.

What Challenges Do We Face in the Production of Biosurfactants by Solid-State Cultivation?

From the previous section, it is clear that much remains to be done in the development of solid-state cultivation systems for the production of biosurfactants. Many different issues will need to be addressed. Some of the most important ones are discussed in the following subsections. The discussion presented in these subsections is quite concise. Anyone with an interest in developing a large-scale solid-state cultivation system and who does not have an in-depth understanding of the technology is strongly advised to read the book of solid-state cultivation bioreactors of Mitchell et al.[17]

Bioreactor Selection

What will be the best type of bioreactor to use for the production of biosurfactants? Mitchell et al[17] discuss in depth the various types of bioreactors available for solid-state cultivation but, in a simple analysis, we might consider the following "typical" bioreactors:

i. Tray or bag systems. In these bioreactors a relatively thin layer of substrate is contained within a tray or a plastic bag. Each tray or bag contains several kilograms of substrate. A large number of trays and bags is placed in a room and conditioned air is circulated around the tray but is not blown forcefully through the bed. The substrate may be left untouched or may be mixed daily by hand. Note that laboratory studies in Erlenmeyer flasks, such as those undertaken by Das and Mukherjee[23] and Krieger et al,[24] correspond to this kind of system.
ii. Rotating drums. In these bioreactors a horizontal drum is filled to about 20 to 30% of its volume with the substrate and then rotated continuously to agitate the bed. Conditioned air is blown into the headspace of the drum but is not forced through the bed itself.
iii. Packed-beds. In these bioreactors the substrate sits on a perforated base plate in a column. Air is blown forcefully through the bed. Typically the bed is not agitated; however, it is possible to have infrequent mixing. Note that while the bed is static significant temperature and moisture gradients can occur within it[17] and that this can lead to significant gradients in growth and product formation. However, the few authors who have studied biosurfactant production in packed-bed bioreactors have not addressed this issue.[12,26]
iv. Agitated and aerated bioreactors. These bioreactors may be of various different designs but are characterized by the fact that agitation is continuous or frequent and that air is blown forcefully through the bed.

The major considerations in choosing a bioreactor for a particular process are the capital and operating costs and the effectiveness of heat removal and moisture control while minimizing damage to the microorganism. We can expect heat removal to be a significant challenge: Bacteria tend to grow reasonably fast, so we can expect high rates of production of waste metabolic heat. In bioreactors other than tray/bag bioreactors the air stream plays a major role in heat removal, since at large scale removal of heat from solid beds to water jackets or cooling coils is not efficient. Therefore we can expect that aeration rates will be determined by cooling requirements and not by oxygen requirements. In other words, the aeration rates required for cooling will be more than sufficient to provide oxygen to the particle surface.[17]

We might expect processes for biosurfactant production to be quite large (for example, based on our own results, 100 metric tons of rhamnolipid biosurfactant would require of the order of 10000 metric tons of fermented substrate). Although it is not impossible to operate tray systems at this scale, it would probably be more cost effective to use other bioreactor types. Note that the majority of solid-state cultivation processes involve filamentous fungi and the damage caused to fungal hyphae when a bed is mixed is often an important consideration in selecting a bioreactor and operating mode. In the case of biosurfactants, the most interesting processes involve bacteria, which are much less susceptible to mechanical damage in an agitated bed of solids. In this case, it is quite probable that "agitated and aerated bioreactors" will be the most appropriate, as these allow the most efficient heat removal, facilitate the addition of water (it can be sprayed as a fine

mist onto the bed surface during agitation) and maintain relatively homogeneous conditions throughout the bed.

What Will Be the Best Substrate to Use?

Will it be possible to use oil-rich meals as the solid substrates? If so, this could reduce substrate costs significantly. However, it needs to be demonstrated whether high yields will be obtained on such substrates or not. The strategy of simply absorbing a nutrient medium used in liquid culture onto a solid support, the strategy used by Krieger et al,[24] should also be considered.

Note that it will not be sufficient to prove that a particular substrate (and the method of preparing it, such as chopping or grinding etc.) promotes high yields in laboratory scale studies. It will also be important to ensure that any such substrate is "well-behaved" within a bioreactor. This means that:

- The substrate is not so sticky that it will produce a cohesive mass when it is agitated within a bioreactor. If this happens, then the bed will lack interparticle spaces for the flow of air (which is important for supplying oxygen). In the case of biosurfactants this is an important consideration, since oils or glycerol will quite often be present in the substrate in order to act as an inducer of biosurfactant production and can increase the cohesiveness of the particles.
- The substrate at the bottom of the bed will not compact under the weight of overlying substrate. If this happens, the interparticle spaces in this region will disappear.
- The particle size is not so fine that it provokes high pressure drops when air is blown forcefully through the bed.

Note that it is not sufficient to characterize the original substrate. The properties of the substrate can change significantly during the cultivation.

Downstream Processing

It is interesting to consider whether the solid might be used directly in some applications at the end of the process. For example, it is conceivable that the solids could be mixed in with soil during bioremediation treatments. In this case all that would be necessary would be to dry the solids.

For those processes in which it is desired to extract the biosurfactant from the solids, it will be necessary to determine the most efficient extraction method. Several issues need to be addressed. Firstly, what extraction method should be used? Krieger et al[24] extracted the rhamnolipid biosurfactant of *Pseudomonas aeruginosa* with water and then undertook a liquid-liquid extraction into chloroform. Possibly liquid extraction is the most feasible method, but work still needs to be done to determine the best solvent system. Secondly, in what mode should an extraction system be operated? Will batch extraction be sufficiently efficient, or will it be necessary to use a counter-current extraction system? Obviously at large scale it will be necessary to recover and recycle the solvent. Note that these issues have received some attention for other products of solid-state cultivation processes, such as enzymes, but not for biosurfactants.[27] Supercritical fluid extraction is also a possibility. It has been studied for recovery of other types of products from the solids at the end of solid-state cultivation processes, but has not been studied for the recovery of biosurfactants.

In the case that the biosurfactant is extracted from the solids, it is important to consider what will be done with the residual solids. In our laboratory we have used sugar cane bagasse in up to four sequential cultivations, without significant loss of process productivity. However, this strategy may have worked due to the fact that the function of the bagasse is to provide an inert solid support. It might not be successful when the nutrients for growth are provided by the original solid substrate.

Note that there is another potential use for the residual solids. The addition of biosurfactants has been shown to enhance the production of some enzymes in solid-state cultivation.[28] Possibly solids from which the biosurfactant has been extracted could be used as a substrate or at least a solid support (with the addition of an impregnating nutrient solution) for a subsequent process for enzyme production.

Monitoring of the Cultivation Process

There are some important issues to be addressed as to how to monitor a solid-state cultivation process for the production of biosurfactant.

Determination of microbial growth in solid-state cultivation systems is not straightforward. This is especially the case in processes involving filamentous fungi, since the fungal hyphae typically penetrate into the substrate, forming a tight association between the biomass and residual substrate, making it impossible to separate and determine the dry mass of biomass. In biosurfactant-producing processes that involve bacteria, there is the possibility of dislodging the bacteria from the solids and trying to determine their amount or number in some manner. However, at best this is likely to give only a coarse indication of growth, since recovery of cells is likely to be partial and solid matter may also be extracted from the residual solid substrate. Note that for the purpose of deciding how to design and operate the bioreactor it is probably more important to characterize the oxygen uptake kinetics than to put a lot of effort into measuring the biomass itself.[17] This is because heat production is directly related with oxygen consumption and heat removal is one of the major factors guiding bioreactor design and operation.

Various methods can be used to monitor biosurfactant production during the process. It is of course possible to remove reasonably large samples and extract the biosurfactant in the same manner as one would in a preparative process. However, it will probably also be possible to develop HPLC methods to monitor the levels of biosurfactants, although this may be complicated by the fact that some biosurfactants are not pure compounds but rather mixtures of congeners. Some authors have monitored the process by adding samples of the fermented solids to water and determining the reduction of surface tension. However, this is an extremely coarse method that does not give a real indication of the amount of biosurfactant. The surface tension measured in these assays tends to fall sharply to a certain value (which corresponds to the surface tension at the critical micellar concentration of the biosurfactant) early in the process and then to remain constant at this value despite the fact that the real biosurfactant level, as measured by some other method, is still increasing significantly.

Conclusion

Solid-state cultivation is an interesting technique for the production of biosurfactants and might make commercial production processes economically viable. However, there is an urgent need to undertake the studies to improve production and to demonstrate the feasibility of processes at pilot scale.

Acknowledgements

Nadia Krieger and David Mitchell thank CNPq, a Brazilian agency of the advancement of science and technology, for research scholarships. Doumit Camilios Neto thanks CAPES, a Brazilian agency for the capacitation of researchers, for a doctoral scholarship. The authors are grateful to CNPq and the company Corn Products Brazil for financing their work in the area of biosurfactants.

References

1. Karanth NGK, Deo PG, Veenanadig NK. Microbial production of biosurfactants and their Importance. Curr Sci 1999; 77:116-126.
2. Makkar RS, Cameotra SS. An update on the use of unconventional substrates for biosurfactant production and their new applications. App Microbiol Biotechnol 2002; 58:428-434.
3. Nitschke M, Costa SGVAO, Contiero J. Rhamnolipid surfactants: Na update on the general aspects of these remarkable biomolecules. Biotechnol Progr 2005; 21:1593-1600.
4. Nitschke M, Costa SGVAO, Haddad R et al. Oil waste as unconventional substrate for rhamnolipid biosurfactant production by Pseudomonas aeruginosa LBI. Biotechnol Progr 2005; 21:1562-1566.
5. Costa SGVAO, Nitschke M, Haddad R et al. Production of Pseudomonas aeruginosa LBI rhamnolipids following growth on Brazilian native oils. Process Biochem 2006; 41:483-488.
6. Lang S. Biological amphiphiles (microbial biosurfactants). Curr Opin Colloid Interface Sci 2002; 7:12-20.

7. Batista SB. Bactérias de ambientes contaminados com petróleo ou derivados produtoras de surfactantes e emulsificantes. Dissertation, Universidade Federal de Viçosa 2002;1-32.
8. Kosaric N, Cairns WL, Gray NCC. Introduction: Biotechnology and the surfactant industry. In: Kosaric N, Cairns WL, eds. Biosurfactants and Biotechnology. New York: M Dekker, 1987:163-181.
9. Mukherjee S, Das P, Sen R. Towards commercial production of microbial surfactants. Trends Biotechnol 2006; 24:509-515.
10. Zajic JE, Seffens W. Biosurfactants. Crit Revi Biotechnol 1984; 1:87-107.
11. Jacobucci DFC. Estudo da influência de biosurfactantes na biorremediação de efluentes oleosos. Dissertation, Universidade Estadual de Campinas 2000;1-117.
12. Veenanadig NK, Gowthaman MK, Karanth NGK. Scale up studies for the production of biosurfactant in packed column bioreactor. Bioprocess Eng 2000; 22:95-99.
13. Lee BS, Kim EK. Lipopeptide production from Bacillus sp. GB16 using a novel oxygenation method. Enzyme Microb Technol 2004; 35:639-647.
14. Yeh MS, Wei TH, Chang JS. Bioreactor design for enhanced carrier-assisted surfactin production with Bacillus subtilis. Process Biochem 2006; 41:1799-1805.
15. Deshpande NS, Barigou M. Performance characteristics of novel mechanical foam breakers in a stirred tank reactor. J Chem Tech Biotechnol 1999; 74:979-987.
16. Vadar-Sukan F. Foaming: Consequences, prevention and destruction. Biotechnol Adv 1998; 16:913-948.
17. Mitchell DA, Krieger N, Berovic M. Solid-state fermentation bioreactors: Fundamentals of design and operation. Heidelberg: Springer 2006:1-450.
18. Ohno A, Ano T, Shoda M. Production of antifungal antibiotic, iturin in a solid state fermentation by Bacillus subtilis NB22 using wheat bran as a substrate. Biotechnol Lett 1992; 14:817-822.
19. Ohno A, Ano T, Shoda M. Production of the antifungal peptide antibiotic, iturin by Bacillus subtilis NB22 in solid state fermentation. J Ferment Bioeng 1993; 75:23-27.
20. Ohno A, Ano T, Shoda M. Production of a Lipopeptide Antibiotic, Surfactin, by Recombinant Bacillus subtilis in solid state fermentation. Biotechnol Bioeng 1995; 47:209-214.
21. Ohno A, Ano T, Shoda M. Effect of temperature on production of lipopeptide antibiotics, iturin A and surfactin by a dual producer, Bacillus subtilis RB14, in solid-stage fermentation. J Ferment Bioeng 1995; 80:517-519.
22. Ohno A, Ano T, Shoda M. Use of soybean curd residue, okara, for the solid state substrate in the production of a lipopeptide antibiotic, Iturin A, by Bacillus subtilis NB22 Process Biochem 1996; 31:801-806.
23. Das K, Mukherjee AK. Comparison of lipopeptide biosurfactants production by bacillus subtilis strains in submerged and solid state fermentation systems using a cheap carbon some industrial applications of biosurfactants. Process Biochem 2007; 42:1191-1199.
24. Krieger N, Mitchell DA, Camilios Neto D et al. Inventors; Universidade federal do paraná (UFPR), assignee. Produção de ramnolipídeos por fermentação em estado sólido. Patent 2007:PI0701366-3.
25. Meira JA. Produção de biosurfactantes por fermentação no estado sólido e desenvolvimento de aplicações para tratamento de solos contaminados por hidrocarbonetos. Dissertation, Universidade Federal do Paraná 2007;1-94.
26. Martins VG, Kalil SJ, Bertolin TE et al. Solid state biosurfactant production in a fixed-bed column bioreactor. Z Naturforsch C 2006; 61:721-726.
27. Lonsane BK, Kriahnaiah MM. Product leaching and downstream processing. In: Doelle HW, Mitchell DA, Rolz CE, eds. Solid State Cultivation. London: Elsevier, 1992:147-171.
28. Kristensen JB, Börjesson J, Bruun MH et al. Use of surface active additives in enzymatic hydrolysis of wheat straw lignocelluloses. Enzyme Microb Technol 2007; 40:888-895.