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Studies on Pelleted Form of Growth Morphology Achieved by *Aspergillus* strains with Different Sugar Treatment Under Submerged Cultivation

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Filamentous fungal fermentation is widely used to commercially produce useful products such as organic acids, enzymes, antibiotics etc. Fungi can be grown in submerged cultures in several different morphological forms: suspended mycelia, clumps, or pellets. A change in fungal morphology is influenced by medium composition, inoculum, pH, medium shear, additives (polymers, surfactants, and chelators), culture temperature, and medium viscosity. Inoculum size is generally recognized as of great importance to the process of fungal pellet formation. Many industrially significant microbial products are produced during secondary metabolism by fungal pellets. Morphology of the fungal pellets has a significant influence on the mass transfer and turn over processes in submerged cultures. In pellets with a loose hyphal structure, convictive transport is possible, but does not necessarily result in higher conversion rates compared to compact pellets morphology. The interaction of hyphae is important in determining the possibility of pellets formation .The pellet diameter and compactness were affected by the agitation intensity of the broth. It has been concluded that fungal growth in pellet form is a favorable alternative which benefits most of the fungal fermentations due to better mass and oxygen transfer into the biomass and lower energy consumption for aeration and agitation. This paper describes the pellets morphology achieved by different fungal strains under culture (PDB) and the production media(with varying sugar components) at 30°C and 180 rpm.

Key words: Pellets, Fungal strains, Growth morphology, Pellet diameter, Mass transfer.

Filamentous fungi are of great industrial importance as producers of therapeutic proteins, insecticides and herbicides, enzymes, organic acids and antibiotics.They are also effective hosts for the production of recombinant proteins. Fungi can be grown in submerged cultures in several different morphological forms such as suspended mycelia, pulpy, clumps, or pellets[1,2].Fungi in submerged cultures often grown in the form of compact spherical masses of mycelium known as pellets .It is reported that pellets consists of an outer shell of growing hyphae and an inner mass of non growing mycelium. The thickness of the outer growing layer of pellets is considered to be limited by nutrient diffusion rate[3].Pellets are classified into smooth and hairy forms.In both cases, the core of the pellet is identified and its shape and size characterized[4].Fungal pellets are a core of densely packed hyphae, surrounded to greater or lesser exent by a more annular dispersed or hairy regions containing radially growing

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hyphae, which can varies in size between several hundred micrometers to several millimeters.The filamentous growth characteristic creates a number of process engineering problems attributed to the morphological change accounted during the fermentation process in large scales such as supply of nutrients, specially oxygen, and the ease of mixing of the broth. Pellets are highly entangled dense masses of hyphae[5-7].

Background Information

In this section we are trying to discuss about the important parameters related to the pellet morphology.

Factors affecting pellet morphology

A change in fungal morphology is influenced by medium composition, inoculum, pH, medium shear, agitation, additives (polymers, surfactants, and chelators), culture temperature, and medium viscosity. For individual strains, each factor has different importance in the growth morphologies; some strains such as *Rhizopus* sp. need strong agitation to form pellets, while some strains such as *Penicillium chrysogenum* require high pH. Inoculum size is generally recognized as of great importance to the process of fungal pellet formation[1]. There are some *Aspergillus* strains where pellets formation were reported at all spore concentration[8].The comparison of the different fermentations showed that the size of the inoculums viz the spore concentration played an important role in the fungal morphology .Literature reported that a high inoculum spore concentration leads to filamentous mycelium ,while low concentration produce pellets with *T.reesei* ,by increasing the inoculum size ,smaller pellets were produced. The structure of the pellets is likely to be species dependent[5]. At concentration of inoculums 10^4 and 10^5 spores / ml,where pellets form predominants ,whereas concentration of 10^8 and 10^9 spores /ml mycelia form developed in dispersed morphology[9]. Agitation also affects the morphology of the fungal systems .Literature indicates that intensive agitation altered the morphology of *Aspergillus* strain and reduced â-glucosidase activity, shearing forces may disrupt fragile microbial tissue and have a marked influence on enzyme production. This effect and difference in the morphology due to higher agitation rates has been reported for other filamentous fungi[5,8].The pellet diameter and compactness were affected by the agitation intensity of the broth; however, the total biomass productivity was not affected. In more intensely agitated stirred tanks (≥600 rpm; impeller tip speed of ≥2.03 m s^{-1}), the stable pellet size was only about ≤ 900 µm. The biomass concentration and the pellet diameter were the main factors that influenced the flow index and the consistency index of the power-law broths[10]. Feng *et al*[11] investigated the effect of fungal pellet size on the the high yield production of destruxinB by *Metarhizium anisopliae* and reported that larger or very smaller pellets size gave poorer yield.Fungal morphology was significantly affected by power input variations .Fungal pellets formed at low speed agitation reached an average pellets diameter of 1500µm and at high speed agitation only 24µm[12]. Distincts pellets with a hairy hyphal layer developed in low speed agitated cultures while micropellets formed at high power input were closely embedded in a filamentous network[5].Different chemicals present in the medium are strongly influenced the fungal morphology.A study of different nitrogen sources on *R. oryzae* ATCC 20344 showed that peptone produced much smaller and more unique pellets than other nitrogen sources such as urea.Metal ions were found to have a significant negative effect on pellet formation while soybean peptone had a positive effect. In addition, potato dextrose broth and calcium carbonate were beneficial to *R. oryzae* for growing small, smooth pellets during the culture[1]. Marina and Geoffrey investigated the influence of clay minerals on the morphology of mycelial pellets. It was found that the inclusion of clay minerals (bentonite, and kaolinite) in the liquid medium influenced size, shape and structure of the mycelial pellets produced. For *C. herbarum*, an increasing concentration of bentonite changed the normal pelleted growth form to diffuse star-like growth, which was also observed for *C. cladosporioides* in the presence of kaolinite. It was found that the clay particles were involved in the formation of pellet structure at all stages of fungal growth[13]. In order to control culture morphology to allow effective long-term perfusion culture, an anionic polymer carbopol (carboxypolymethylene) at 0.1% was added to the culture medium to promote growth in a more dispersed form. It has been

observed that this polymer promote a more dispersed pellet growth in fungal cultures such as *Aspergillus niger* and *Rhizopus arrhizus*[2].

Mechanism behind the pellets formation

Inoculum size is generally recognized as of great importance to the process of fungal pellet formation. Generally, the interaction of hyphae is considered as the main force in forming clumps. In the early stage of growth, the higher the inoculum size, the more interaction with the hyphae, thereby preventing the development of pellets and the more possibility the clump will be formed. Thus, it has been concluded by other researchers that low inoculum concentrations are beneficial for pellet production[1,10].Whereas other researchers have been suggested that the formation of pellets originated from the adherence of germinated spores to solid particles in medium. The attached solid particles were also digested during the fungal fermentation and resulted in the formation of the smooth and hollow pellets[14].The gyratory movement of the shaker promoted the formation of uniform fungal pellets. The main reasons of a different rheological behavior of pellets suspension are freely dispersed mycelia. The lower biomass concentration at higher energy input is related to higher shear forces, which leads to strong erosion processes and tends to structural change in the pellets morphology[2].High shear stress can cause breakage of filaments .A cycle of mycelia fragmentation and regrowth has been observed in *Aspergillus niger* .Fragmentation and regrowth are beneficial for the product formation because the mycelia that are most susceptible for the fragmentation are old and heavily vacuolated parts of the filaments that are metabolically inactive .,the new tips generated from fragmentation give rise to new filaments[9].

Models related to pellets morphology

Many industrially significant microbial products are produced during secondary metabolism by fungal pellets. Most models of mycelial pellet growth, however, consider only the primary growth phase[15].Productivity of the filamentous fungi like *Aspergillus niger* ,is closely connected with the occurring morphological development based on macroscopic growth[16]. Emerson developed first model describing pellets growth .It describes the increase in the biomass of one pellets starting with the mass of one pellet.

$$M^{1/3} = Mo^{1/3} + kt$$

This is also called cube root law is based on a growing outer pellet shell. By assumption of the ideal spherical pellets and the constant pellets density Pp the constant k can be calculate by using the pellets diameter in the beginning ro.

$$k=(r-ro)\sqrt[3]{4/3\pi pp}/t$$

The thickness of the assumed active biomass layer is mainly determined by diffusion of the limiting nutrient like O_2 and depends like the pellets density. Three general pellets break up mechanisms in stirred tank reactors are known.a)fluid induced shear stress by eddies ,b)particle–particle collisions, c)particle–impeller(or baffle)collisions. Two general types of pellets fragmentation are conceivable .Direct rupture of whole pellets and the erosion process .Erosion process leading to the chipping off of hyphae from the surface is predominant mechanism and leads to very small fragments which may reseed pellets population[17]. High energy dissipation leads to lower biomass concentration within the bioreactor ,whereas decreasing energy input promotes biomass growth. Higher shear stress leads to smaller and more dense pellets, in contrast to big and fluffy ones at lower energy inputs.The pellets structure plays an important role with regard to the active layer of the pellets and the amount of active biomass .Due to the increasing shear stress ,the pellets grow slower ,which leads to smaller and more dense pellets[16].

Engineering aspects of pellets morphology

Hille *et al*[18] investigated the oxygen profiles and biomass distribution of biopellets of *Aspergillus niger* .Mass transport in fungal pellets such as *Aspergillus niger* is strongly dependent upon the pellet morphology .There are many reports in literature that biomass is not distributed homogenously over the pellet radius.The distribution of biomass inside the pellet and the shape of the oxygen profile change significantly over the cultivation time and dependent on the

culture conditions .The influence of hydrodynamic conditions on mass transfer at the bulk/pellet interface and inside the pellet is high. In pellets with a loose hyphal structure ,convctive transport is possible, but does not necessarily result in higher conversion rates compared to compact pellets morphology[18]. In the pellet type of morphology mass transfer at the liquid–solid interface and transport within the pellets are the bottle necks in biomass specific productivity. The thickness of the outer growing layer of pellets is considered to be limited by nutrient–diffusion rate. Thus the mycelium inside the outer growing shell of the pellet will be anaerobic, so called cube root growth of fungi. Thus it is concluded that there is the large differences between the metabolism of the outer and the inner hyphae of the pellets[3].

Significance and limitations of pellet morphology

Hermersdoerfer *et al*[19] reported that the number process engineering problems arises due to filamentous nature of the growth which affects on productivity. Excessive hydrodynamic shear stresses are known to damage mycelial hyphae and pellets, but much lower shear stresses are sufficient to influence growth morphology. In such fermentations, the mass transfer of oxygen and nutrients is considerably better and the subsequent separation of the pellets from the medium is simpler. Since agitation and aeration is also much easier in such a system, the power input therefore the operating cost is lower[6,7]. Raviraja stated that pellet size affects mycelial ergosterol content in aquatic hyphomycetes. There was a significant, positive correlation between the amount of ergosterol per unit mass and pellet diameter[20]. The possibility of the pellet formation despite the presence of the high content of suspended solids would be of great advantage to perform the treatment process and the fungal biomass production on the airlift-type bioreactors by lowering medium viscosity and better mass exchange of oxygen and nutrients[14]. In addition to giving a more desirable rheological characteristic and higher oxygen transfer, it is more advantageous to grow the fungal cells as pellets than mycelial mats from the cell retention stand point. There are however technical barriers need to be overcome so that the fungal pellets do not become oversized in the long-term culture rendering mass transfer and mixing problems**.** The large pellet size and high biomass concentration impaired culture circulation in the air-lift bioreactor which affects the overall productivity[2].

MATERIAL AND METHODS

Aspergillus niger NCIM 777 and *Aspergillus fumigatus* NCIM 902 were procured from National chemical laboratory (NCL), Pune. Fungal spores from a stock, kept at 4° C in 20% (v/v) glycerol. All cultures were grown on potato dextrose agar (PDA) slants and petriplates at 28° C for 5-6 days. Slants were maintained at 4°C and subculture about monthly intervals. Experiments have been performed in a 250 ml Erlenmeyer flasks containing 150 ml of Potato dextrose broth(PDB) (In g/l Peeled Potato, 200; Dextrose, 20; and Yeast extract,0.1) medium in which 5 loopfull cultures of fungal spores or conidia was added and shaken at 180 rpm at 30° C in an incubator shaker for 96h.While other sets of experiments were carried out usingmediacontaining, In(g/l) Urea, 0.3; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0;$CaCl_2.2H_2O$,0.4; $MgSO_4.7H_2O$,0.3; Peptone,1.0; Tween80,0.2; $FeSO_4.7H_2O$, 0.005;$MnSO_4.7H_2O$, 0.0016; $ZnSO_4.7H_2O$,0.0014;$CoCl_2.6H_2O$,0.02.and lactose, sorbitol, xylose used as carbon source separately in different sets of experiments[21]. The autoclaved media were inoculated with ~3 ml PDB culture solution of the respective microorganisms by maintaining same cell dry weight.

RESULTS AND DISCUSSION

From the above observations we found that *Aspergillus niger* showed their growth as medium sized uniform, spherical compact pellets form in the potato dextrose based culture broth media whereas *Aspergillus fumgatus* achieved somewhat bigger nearly round shaped diffused, fluffy branched, nonuniform mycelial pellets. Fig 1. and 2.describes growth of *Aspergillus niger* and *Aspergillus fumigatus* in the potato dextrose broth based culture media.

As the literature suggested that PDB (Potato Dextrose Broth)is a better carbon source for pellet formation of *R. oryzae* when compared

to glucose. It has been found by this study that PDB had a large impact on pellet size such as pellet diameter, total biomass, and total amount of pellets. This means that the vitamins in PDB might be the main substances causing the difference on fungalsize[1].

In other sets of experiments where lactose, xylose and sorbitol act separately as carbon source in the production medium, *Aspergillus niger* and *Aspergillus fumigatus* attained different pelleted forms **.** *Aspergillus niger* showed their growth in the form of big pellets with lesser in number in the lactose based production media whereas they grown as big pellet with more in number in the xylose and sorbitol based production media as shown in Fig 3-5. On the other hand *Aspergillus fumigatus* achieved their growth as in the form of smaller and medium sized pellets but their number varies according to the media used such as in lactose and xylose based production media pellets were more in number while lesser in sorbitol based production media as shown in Fig 6-8. Results indicates that the number and size of the pellets varies by same microorganisms in the culture and production media.

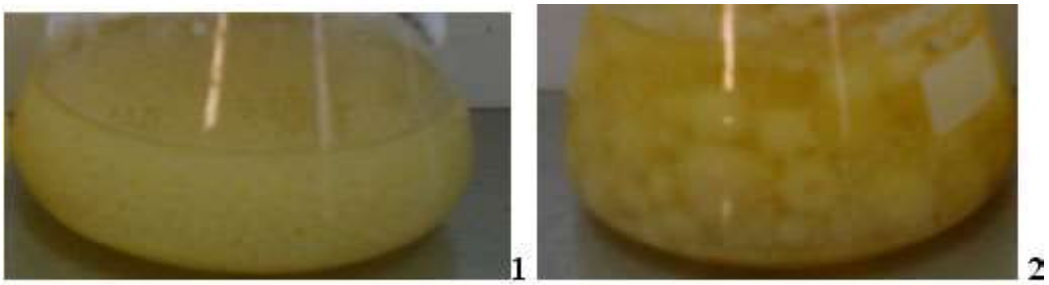

Fig. 1-2. Growth of *Aspergillus niger* and *Aspergillus fumigatus* on Potato Dextrose broth media at 180 rpm and 30°C

Fig. 3-5. Growth of *Aspergillus niger* on lactose, xylose and sorbitol based production media respectively at 180 rpm and 30°C

Fig. 6-8: Growth of *Aspergillus fumigatus* on lactose, xylose and sorbitol based production media respectively at 180 rpm and 30°C

Aspergillus niger grown as medium sized pellets in PDB culture media whereas they formed big pellets in the sugars based production media whereas *Aspergillus fumigatus* grown as big non uniform pellets in the culture media whereas in different sugars based production media they achieved very small pellets or bead form of growth. .It has been suggested from the above observation that lactose,xylose and sorbitol based production media are more favorable for pellets growth(pellet size) in *Aspergillus niger* while much effective for pellets formation (pellets number) in *Aspergillus fumigatus*.. whereas for *Aspergillus niger,* potato dextrose based culture media are much more efficient for pellets formation (pellets number) rather than pellets growth.Due to all the experiments have been carried out at same temperature (30°C) and same rpm (180) therefore components present in the media (production or culture) plays a vital and lead role in the formation of various pelletized form. Media composition and mainly sugars are the main factors which directly affects the pellets morphology (number,size and shape).This might be due to the sugars uptake capability of the microorganism by membrane transport system. Efficiency of sugars uptake by microorganisms differ for different sugars present in the media due to their biochemical complex city[22].As literature reported that filamentous growth characteristic creates a number of process engineering problems attributed to the morphological change accounted during the fermentation process in large scales which affects on the maximum product or productivity therefore pellatized morphology is more suitable for industrial fermentation.

CONCLUSION

It has been concluded that fungal growth in pellet form is much favorable alternative which benefits most of the fungal fermentations since it not only makes repeated-batch fungal fermentation possible but also significantly improves the culture rheology which results in better mass and oxygen transfer into the biomass and lower energy consumption for aeration and agitation which is the ultimate requirement to get efficient fermentation for achieving better productivity. In industrial applications pellet morphology is usually preferred in fermentations and in downstream processing due to the non viscous rheology of the broth. From the above observation we can also infer that the compoments present in media plays lead role in formation of different pelleted forms. Finally this morphological information (pelleted form) may be beneficial in their extensive use for production of some valuable products such as cellulases. By knowing this morphological pattern we can developed a better morphological growth form, which gives higher product yield or productivity.

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