Global Journal of Biotechnology & Biochemistry 4 (2): 75-83, 2009
ISSN 2078-466X

Pellet Morphology, Broth Rheology and Statin Production in Submerged Fermentation of *P. citrinum*

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Abstract: Pellet formation of *P. citrinum* in submerged fermentations in shaken flasks was used to examine fungal morphology and statin production under different conditions. The size and morphology of pellets differed according to the applied cultural conditions. The pellets with loose hairy surface and short mycelium were the ones with high production levels. The number of pellets was inversely related to the production. The rheology of the culture broth at its optimal production conditions (40% aeration and 150 rpm) was compared to that at maximal production conditions (addition of 15µl Triton X-100), the results show a slight decrease in viscosity (1.4 to 1.3 cP), pellet number (490-102) and size (3-5 to 0.5 to 3mm), also in yield stress and flow index while there was an increase in the consistency index under maximal conditions. The non-Newtonian (viscous) culture is required for statin production by *P. citrinum* in submerged cultures. This study does not only show that it is possible to control and regulate pellet morphology to obtain high statin production but also that controlling culture rheology is a key step.

Key words: *P.citrinum* · Morphology · Rheology · Statin production

INTRODUCTION

Submerged culture fermentation is usually preferred in the industrial production of antibiotics, enzymes and organic acids for their application in different fields It is well documented that fungi could have two morphological states; mycelia and pellets, the latter being mostly preferred in the fermentation process for its non-viscous (Newtonian) rheology of the broth [1]. This process helps in the mass oxygen transfer and the nutrient are rendered more available and subsequently the pellet formation and separation from media is considered much simpler. There are many factors which control the pellet formation in a culture, hence, affecting the production of the desired compound; these factors include the type of the strain, pH, aeration, agitation, the nutrients added, inoculation ratio and genetic factors of the culture [1].

Submerged cultures of filamentous fungi are widely preferred to produce commercially important metabolites, among which are the statins [2]. Natural statins are anti cholesterol drugs which are produced as secondary metabolites by a variety of filamentous fungi including *Aspergillus terrus*, *Monascus spp.*, *Penicillium spp.*, *Trichoderma spp*., *Paecilomyces spp*., *Eupenicillium spp.* and *Pleurotus spp*. [3]. The production of statins is governed by a number of factors which control the pellet formation depending on the aeration rate and agitation speed [4]. Pellets are preferred for their non viscous rheology of the broth [5]. There are a number of factors which govern the signaling process involved between fungal morphology and physiology hence, regulating the cell production [6]. A good control of mycelial morphology in fermentation is very important for many industrial applications [7]. There are a number of opposing reports concerning the relation between pellet morphology and maximal production of certain industrially important fermentation yields; for example: pelleted *A.terreus* is preferred for production of itaconic acid while filamentous form is required for the production of pectic enzymes by *A. niger* [1].

Fermentation broth rheology greatly affects the transport of oxygen and nutrients which, in turn, has a strong influence on the efficiency and productivity of the entire fermentation process [8]. Since there is a necessity to equilibrate the production and handling processes in a culture, therefore, there is a need to balance morphology,

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rheology and product formation through regulating the factors which affect the above mentioned requirements.

With this in perspective, the current work focuses on examining the effect of agitation speed and aeration rate on the pellet morphology and statin production in submerged fermentation of *Penicillium citrinum*, examine the use of hydrogen peroxide as a non-conventional source of oxygenation and Triton X-100 as a mode of increasing dispersion and compare the optimal and maximal statin production in terms of pellet morphology and broth rheology.

MATERIAL AND METHODS

Microorganisms: The screening experiment for statin production was carried out using different fungal strains belonging to *Aspergillus terrus*, *Monascus purpureus* and *Penicillium citrinum*. *Candida albicans* was used for statin bioassay. These strains were kindly provided by "Assuit University Mycological Center", Faculty of Science, Assuit University, Assiut-Egypt. Fungal cultures were monthly cultured on PDA and stored at 4°C until used.

Preculture and Submerged Cultivation: For spore suspension preparation, 5ml of sterilized saline was added to a 10-day slant of fungal strains. The surface was scratched with an inoculation needle to obtain the fungal spore suspension. The concentration of the spore suspension was adjusted at 1×10^8 spores/ml.

The inoculum was prepared by transferring 1ml of the spore suspension to 50ml sabroad broth medium containing 2% glucose and 1% peptone (pH 5.5) and incubated at 28 ±1 °C for 24h under shaking conditions (150 rpm).

A 15% (v/v) inoculum was transferred into the production media previously described for statin production [9]. Fermentation was carried out in 250ml Erlenmeyer flasks. Cultures were incubated at 28 ±1 °C for 10 days on a rotary shaker at 150 rpm.

Statin production was determined at different volumes of cultivation media to test aeration (50, 75, 100, 125, 150 ml working volume in 250 ml Erlenmeyer flasks), agitation speeds (100, 150, 200 rpm), different H_2O_2 concentrations (5, 10, 15, 25, 50, 100, 150 µM) and Triton-X 100 concentrations (5, 10, 15, 20, 30, 40, 50 µM).

Statin Extraction and Estimation: At the end of the cultivation time, 20ml of the whole broth culture was acidified to pH 3 with 2N phosphoric acid and extracted for three times with an equal volume of methanol under shaking conditions (200 rpm) for 20 min [10]. The combined extracts were dried over sodium sulfate anhydrous and concentrated to a final volume of 2ml. Statin was estimated by a rapid procedure based on the anti-fungal properties of statin against the yeast *Candida albicans*, the statin activity was calculated from the inhibition zone by an equation was reported by Vilches Ferrón *et al.* [11].

Fungal Growth and Morphological Characterization: The fungal pellet morphology was characterized by measuring the changes of the following parameters (1) pellet diameter, (2) number of pellets and (3) visual observation of the pellet compact shape, structure and color. Ten milliliter of each whole broth culture was decanted into sterile plate and the obtained pellets were washed twice with distilled water. Pellet density can be measured by visual observation which is equal to the number of pellets multiplied by the dilution factor. Pellets were placed on a glass slide for morphological characterization. The pellets were observed as compact or soft; small or large and hairy or smooth. The size was determined as pellet diameter in millimeters. The change of pellets color was also observed.

Rheological Studies: Rheological parameters were measured using a programmable rotational viscometer (Brooke-field DV-II+ with standard spindle V-72,21.67 mm diameter x 43.33 mm height; Brookfield, Middleboro, MA, USA) for the broth media under the worst (80% aeration), optimal (60% aeration) and maximal (60% aeration and 15µl Triton X-100) statin production conditions.

The theory of rheological measurements with the "cup and vane" rotational viscometer is well known. A rheogram of the culture broth of interest is obtained as a plot of the average shear stress τ_{av} measured at different average shear rate (γ_{av}) values [2]. The plotted data are the mean values of triplicate separate measurements.

RESULTS AND DISCUSSION

Screening: Several fungal species are known to produce statin, a secondary fungal metabolite known for its ability to influence the *de novo* synthesis of endogenous cholesterol. The most common species are *Monascus* [12], *Aspergillus* [13], although *Pencillium spp.*, *Trichoderma spp*., *Paecilomyces spp*., *Eupenicillium spp.* and *Pleurotus spp*. [3] were also reported, yet were not studied as much as the previously mentioned species.

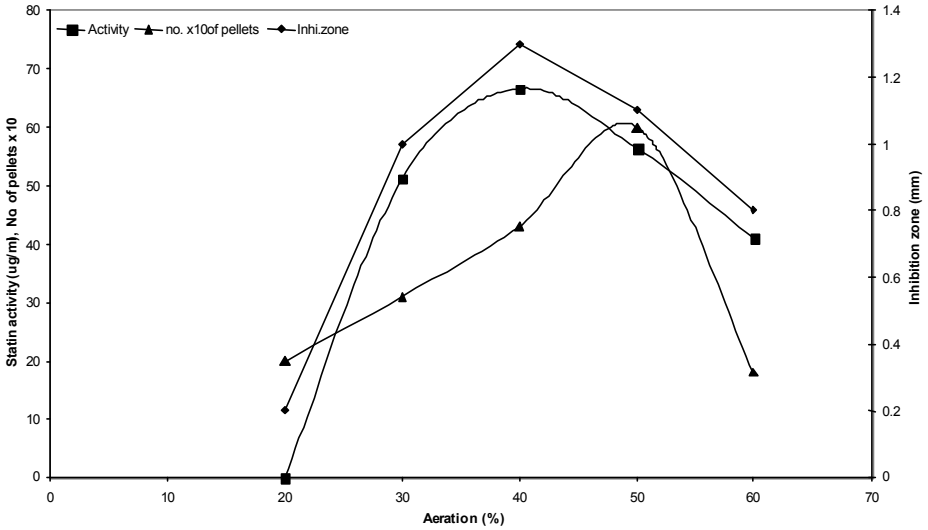


Fig. 1: The effect of aeration (%) on statin activity (µg/ml), number of pellets (x10) and inhibition zone (mm) for *P. citrinum* in submerged fermentation

Table 1: Effect of aeration on the pellet size and morphology of *P. citrinum*

Aeration (%)	20	30	40	50	60
Pellet size (mm)	1-2	2-3	3-5	3-5	4-6
Pellet morphology	Very small				Large
	Compact				Loose pellets
	Minimal growth →				Increase in growth
	Dark pellets				Hairy Light pellets

In the first part of the research, a screening for statin production was carried out using different fungal strains belonging to *Aspergillus terrus*, *Monascus purpureus* and *Pencillium citrinum*. The screening performed by the rapid anti-fungal inhibition against *Candida albicans* showed that *Pencillium citrinum* possessed the highest inhibition zone meaning that it produces the highest levels of statin against two tested *Candida albicans* isolates. Also statin activity was studied using different extraction solvents as well as the crude fungal filtrate containing statin (data not shown). This strain was to be used in the following experiments.

Factors Affecting Statin Production: Since the morphology of filamentous microorganisms is influenced by a number of factors such as the agitator type, agitation speed and dissolved oxygen [8], the research was focused on studying the effect of some factors on the morphological characterization and statin production. Since pellet formation has been related to minerals, carbon or nitrogen sources in the media, as well as the age of the inoculum [14], therefore, the inoculum age, initial pH, media used were fixed during all the upcoming experiments.

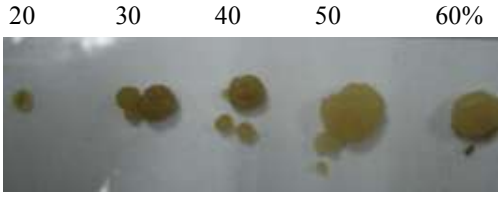


Plate 1: Represents the variation in pellet morphology Under different aeration conditions

Testing the effect of aeration on statin production and pellet morphology, the results shown in Fig. (1) show that there is no correlation between statin production and pellet number. The maximum statin production was observed at 40% aeration which reached a maximum of 66 µg/ml, while the number of pellets reached its maximum at 50% aeration (600/250 ml culture) after which it dropped (180/250 ml culture). While the pellet size shows an increase in the pellet diameter as the aeration increases, reaching the maximum of 4-6 mm at 60% aeration. The pellets also increased in their size and the loose network and hairy pellets were more observed at 60% aeration, the moderate pellet size and morphology was detected at 40% aeration before which the pellets were very small, compact and low in count (Table 1). Plate (1) clearly demonstrates

Table 2: Effect of agitation on the pellet size and morphology of *P. citrinum*

Agitation (rpm)	100	150	200
Pellet size (mm)	0.1-0.7	3-5	0.1
Pellet morphology	Aggregates of 3-4 pellets of various sizes Light Uniform Good growth	Large Loose Light pellets of good growth	Very small pellets but visually don't seem compact

Table 3: Effect of addition of different hydrogen peroxide concentrations (as an unconventional source of oxygen) on the pellet size and morphology of *P. citrinum*

H2O2 (mM)	0	5	15	25	50	100	150
Pellet size (mm)	2-3	0.5-3	0.5-5	1-4	1-5	1-10	1-6
Pellet morphology	small	Individual pellets Dark brown Compact Smooth surface Some loose hairy long mycelium (2-5 mm)	Individual pellets Dark compact Smooth surface Little loose hairy mycelium (0.5-8 mm)	Variable size pellets	Very small Hairy Loose pellets	Variation between low numbers of very big and high numbers of very small pellets with a ratio of 1:40	Variation in color, size and pellet distortion.

the variation in pellet morphology according to the percentage of aeration. It is obvious that the aeration is a strong parameter by which the productivity is affected. It is known that aeration could have a positive or negative effect on the culture productivity through affecting the morphology or cell growth and protein production [15]. Aeration is considered to be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product and oxygen [4]. Oxygen enriched cultures of *Aspergillus terreus* resulted in large fluffy pellets produced high lovastatin titers [2, 16].

The agitation speed is another factor which affects the morphology of filamentous fungi [8], it is considered one of the important factors affecting the pellet formation [7]. In the following experiment, three agitation speeds were studied, the results in Fig (2) show that the optimal statin production was attained at 150 rpm (66.66 µg/ml) and it was inversely proportional to the pellet number, although the variations were not to be considered noteworthy (470, 430 and 480 pellets/250 ml culture at 100, 150 and 200 rpm respectively). There was a discernable variation in the pellet sizes observed at different agitation speeds (0.1-0.7 mm at 100 rpm, 3-5mm at 150 rpm and 0.1 mm at 200 rpm). The pellet morphology was also variable, at 100 rpm the pellets were uniform in shape in the form of 3-4 aggregates, this is probably because the pellets are able to agglomerate at such mild agitation speed, at 150 rpm the pellets were larger and loose. At 200 rpm the pellets were visually very small and detached but they did not seem compact (Table 2). Michel *et al*., 1990 reported that mild agitation of 100 rpm results in a few large pellets while high agitation of 200 rpm resulted in numerous small pellets. In our experiment, the employment of 150 rpm as an intermediate agitation speed between mild and high resulted in obtaining pellets which were separate, loose and bigger in diameter than both the mild and high agitation speed, however, the effect of agitation speed on the number of pellets were contradictory to what Michel *et al.* [17] reported. Borràs *et al.* [7] used an agitation speed of 135 rpm to avoid break-up of the pellets. Controlling the hydrodynamic conditions applied during cultivation was successfully employed on *P. chrysosporium* to obtain pellets of different sizes and hence control the lipid peroxidase activity [18].

The dissolved oxygen is considered an important factor which affects the pellet morphology [8]. In the following experiment, low concentrations of hydrogen peroxide were added to different cultures to obtain an increase in the oxygenation of the culture. The use of low concentrations of hydrogen peroxide was reported as an unconventional method of oxygenation [19]. The results shown in Figure (3) shows that the optimal statin production was attained at 15 and 25 µl reaching 76.2 µg/ml, after which the statin production decreased reaching the lowest production when 150 µl was added (15.4 µg/ml). On the other hand, the pellet numbers increased with the increase in hydrogen peroxide concentrations, reaching a maximum of 2000 pellet/250ml culture when 150 µl was added to the media, comparing to 60-76 pellet/250ml culture for the optimal statin production. The pellet morphology also showed an increase relevant to the increase in hydrogen peroxide added to the media (1-6mm for 150 µl hydrogen peroxide compared to 0.5-5 at 15 µl and 1-4mm at 25 µl). The pellet morphology varied greatly in size, color and compactness and small detached mycelium of 0.5-8 mm were observed at lower concentration of hydrogen peroxide (Table 3).

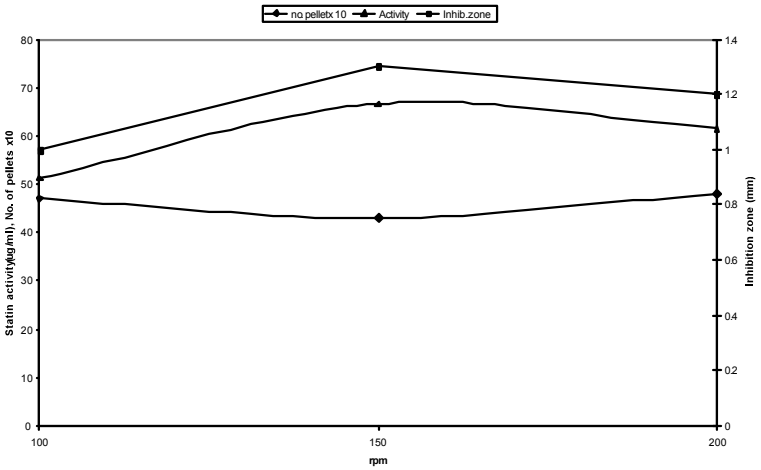


Fig. 2: The effect of agitation speed (rpm) on statin activity (µg/ml), number of pellets (x10) and inhibition zone (mm) for *P. citrinum* in submerged fermentation

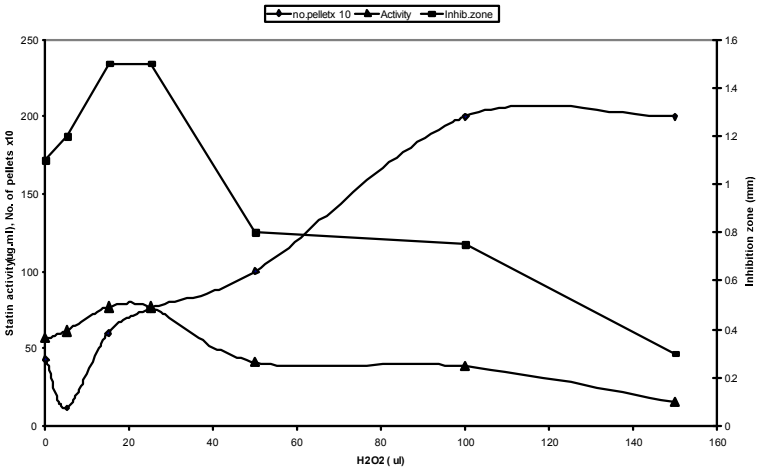


Fig. 3: The impact of adding different concentrations of hydrogen peroxide (µl) on statin activity (µg/ml), number of pellets (x10) and inhibition zone (mm) for *P. citrinum* in submerged fermentation

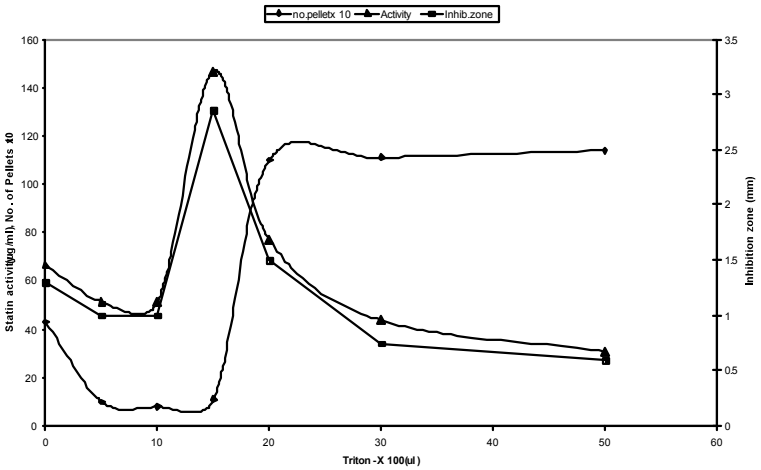


Fig. 4: The impact of adding different concentrations of TritonX-100 (µl) on statin activity (µg/ml), number of pellets (x10) and inhibition zone (mm) for *P. citrinum* in submerged fermentation

Table 4: Effect of addition of different Triton-X 100 concentrations (as a source for decreasing viscosity) on the pellet size and morphology of *P. citrinum*

Triton-X 100 (µl)	0	5	10	15	20	30	50
Pellet size (mm)	3-5	1-3	0.5-3	0.5-3	2-3	1-2	1-2
Pellet morphology	Large Loose light	Creamy white 50% small mycelium Smooth uniform pellets	White and beige colored pellets 75% small mycelium Uniform varying in size very light culture	White and beige pellets 75% small mycelium Uniform pellets	Light Compact hairy	Light compact	Light compact

Table 5: A comparison between morphology, rheology and production under optimal (optimum aeration, agitation) and maximal (15µl Triton X-100) conditions

Parameter	Optimal	Maximal
Viscosity	1.4 cP	1.3 cP
Yield stress	0.84 D/cm^2	0.76 D/cm^2
Flow Index(n)	0.8	0.74
Consistency Index(k)	5.15 cP	7.64 cP
Pellet size	3-5 mm	0.5-3 mm
Pellet number	490	102
Morphology	Medium, compact, loose, hairy	25% uniform, smooth pellets and 75% short mycelium
Production	66	149

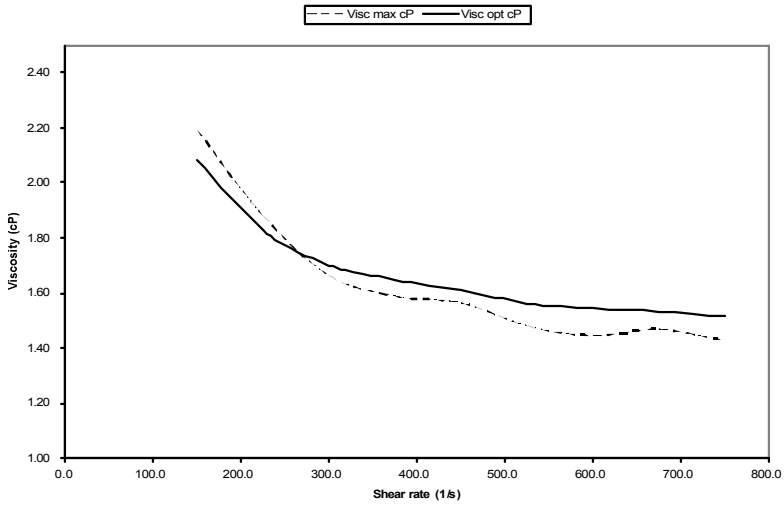
The problems related to the oxygen concentration has been shown to exert a great influence on pellet formation, the use of air-pulsed reactor solved this problem and allowed a better oxygen transfer [7].

Triton X-100 is a surfactant which is added to microbial culture media to decrease the surface tension; its use is considered to increase the bioavailability of the culture media components and the dissolved oxygen. In the following experiment, the addition of different concentrations of Triton X-100 was correlated to statin production and pellet morphology. The results in Figure 4 shows that the addition of 15 µl induced the maximal production of statin (146.66 µg/ml), after which there was a drop which reached 30.8 µg/ml in statin production when 50 µl of Triton X-100 was added to the culture media. On the other hand, the pellet number increased to 1140 pellet/250 ml culture at the lowest statin production compared to 110 pellet/250ml cultures for the maximal statin production. Examining the pellet morphology, the pellet size decreased with the increase in Triton X-100 concentrations and was more compact, while at the maximal statin production, the pellets were variable in size (0.5-3 mm) and about 75% of the culture was short mycelium (Table 4). From this we can depict that the addition of Triton X-100 in the concentration of 15 µl was efficient to decrease the surface tension without damaging the pellet morphology or the statin activity, yet the loose short mycelium also contributed to the production of the maximal statin activity.

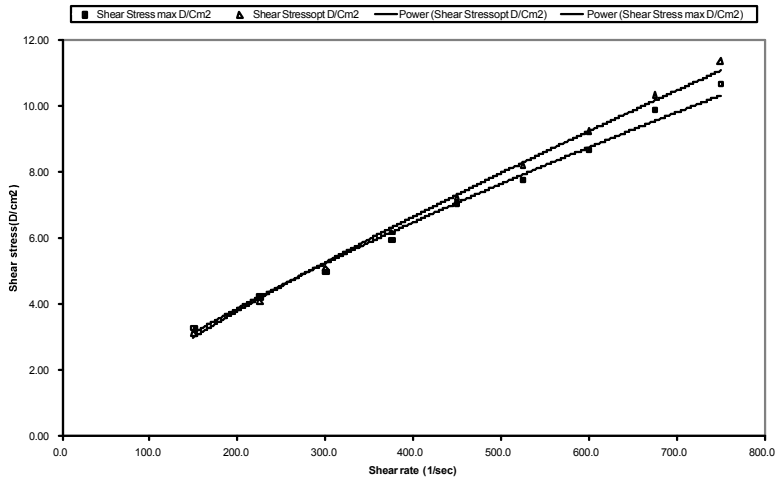
These results show that medium size pellets with medium looseness/compactness is the required shape and morphology for statin production regardless to the pellet count or its large size. The fact that pellet morphology depends on a number of parameters which influence the activity of various extracellular enzymes has been reported before and gained more attention recently [18].

Broth Rheology: Many studies have discussed the pros and cons of growth morphology in terms of different products and have concluded that growth in the form of pellets is favored, not only because of the increased chance of using the fungal batch in fermentation repeatedly, but also for the significant improvement in culture rheology which results in better mass and oxygen transfer into the biomass, besides the low energy consumption needed for aeration and agitation [14]. The use of pellet cultures also solve operational problems related to biomass growth on the bioreactor walls, agitators, probes and baffles [7]. Understanding the broth flow behavior is necessary to develop a design strategy that will help overcome such problems [8].

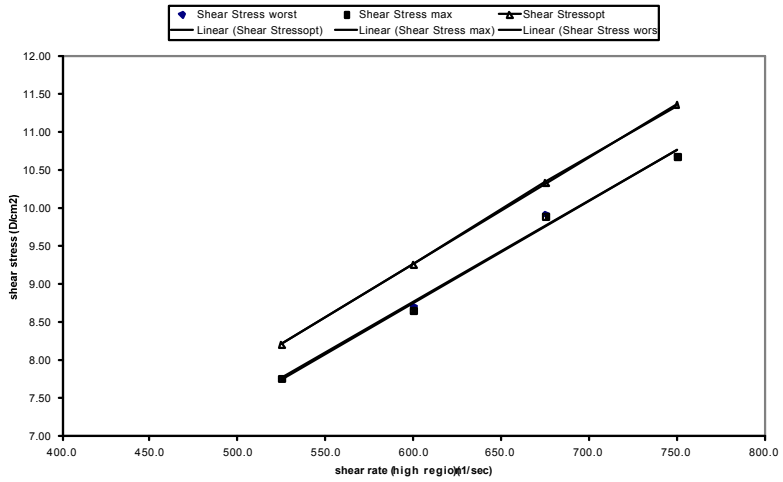
In order to add to an improved description of cell suspension growth, the rheological properties of *P. citrinum* cultivated under optimal and maximal statin production conditions were studied. Figures 5A,B and C represent rheograms of the culture suspension (with pellets) relating the viscosity and shear rate (A) and the relation between shear stress and shear rate in the low (B) and high (C) shear regions.



(a)



(b)



(c)

Fig. 5a,b,c: Rheograms representing the relationships between shear rate and viscosity (a), low shear rate and shear stress (b) and high shear rate and shear stress (c) for pellets of *P. citrinum* at optimal (▲) and maximal(■) statin production conditions

In this study, the power law was used to describe the flow behavior of *P. citrinum* fermentation broth. The consistency index (viscosity in the low shear region) of the fermentation broth can be related to shear rate by the following equation:

$$\mu = K(\gamma)^{n-1}$$

Where μ is the shear stress, K and n are the consistency index and the flow behavior index respectively and γ is the shear rate [8]. The yield stress and viscosity were calculated from the linear fit of the shear stress and shear rate in the high shear region.

The results obtained are summarized in Table (5) which shows that the flow index n is less than 1 in optimal and maximal statin cultivation conditions; this means that the culture exhibits a non-Newtonian flow behavior. The viscosity, consistency index and statin production were higher at maximal conditions, while the yield stress, pellet size, pellet number and flow index decreased. The presence of the loose hairy short mycelium represented approximately 75% of the culture at maximal statin production conditions, while short mycelium was not visible in the optimal statin production conditions. Although pellets decrease the viscosity approaching a Newtonian flow behavior, which in turn results in a successful industrial production [7], yet its is evident from the obtained data that the case is different regarding statin production by *P. citrinum* and that the non-Newtonian flow behavior is required. Raposo and Lima-Costa [20] confirm that the majority of suspension cultures usually exhibit a non-Newtonian behavior, Gogus *et al*. [1] state that a maximal production of polygalacturonase, pellet morphology and low suspension viscosity were correlated with a broth rheology close to Newtonian flow behavior. On the other hand, the need for a non-Newtonian flow behavior in the cultivation vessel could be controlled by morphologically engineering the pellet morphology and broth rheology through the addition of xanthan gum to create fermentation media of artificially high viscosity in order to regulate the collision of the formed pellets [21]. The pellet formation is highly dependent on the degree of aggregation among the fungal hyphae, the hyphal growth results in the formation of a branched structure which facilitates the permanent entanglement of aggregated particles, this account for the three-dimensional structure of microbial pellets [22]. Particle aggregation is dependent on the collision energy, which if not sufficient, the particles will not aggregate. The movement of particles in a turbulent system is dependent on many parameters, among which is the hydrophobicity of the aggregates [21]. In the present study, the addition of Triton X-100 to the media was used for this purpose and also as a mode for increasing the bioavailability of oxygen by decreasing the surface tension to obtain maximal statin production, the freedom of pellet movement is presumed to be facilitated by decreasing the external charges on the fungal walls since Triton X-100 is a non-ionic surfactant known for its ability to strip cell membranes of some or all their charges, depending on their concentration.

CONCLUSION

The importance of achieving the suitable pellet morphology and broth rheology is crucial for obtaining the maximal statin production in submerged fermentation media for *P. citrinum*. There is a direct relationship between the hydrodynamic movement of the pellets in the cultivation media and the loose fungal pellets which obviously require adjusting prior to using the pellets on an industrial scale. Contrary to other fungi, *P. citrinum* was greatly affected by aeration rather than agitation speed. The addition of hydrogen peroxide in low concentrations enhanced the statin production and affected the pellet morphology while the addition of Triton X-100 upscaled the production of about 44% and also affected the pellet morphology. The loose short mycelia present in the culture obviously obtained more nutrients and oxygen than the compact uniform or loose pellets. The non-Newtonian flow behavior aids in controlling the movement of pellets, avoiding the hydrodynamic shear stress. Pellet number is irrelevant to productivity, while medium pellet size of open filamentous morphology were better in producing statin compared to small dense compact pellets. The best statin activity is obtained at oxygen-rich conditions which are not excessively turbulent.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Dr. Omar El Desouky for performing the rheological measurements and Dr. Nabila Selim for her assistance in revising the broth rheology section of the manuscript.

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