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Evaluation of Morphological Properties of *Solanum chrysotrichum* Cell Cultures in a Shake Flask and Fermentor and Rheological Properties of Broths

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Summary

Solanum chrysotrichum cells were grown in a shake flask and a stirred tank bioreactor. Their growth kinetics and rheological and morphological behaviors were evaluated. The specific growth rate obtained in both systems was similar (0.09 day^{-1}), but biomass yield achieved in shake flasks was 60 % higher than that in the fermentor. Culture broths exhibited non-Newtonian and shear-thinning characteristics, which can be simulated by the Ostwald de Waele model (power law). Results show that the pseudoplasticity of culture broths was determined by the biomass concentration and aggregate size distribution, while the aggregate roundness, measured as the elliptical form factor, was similar in both systems. Morphological analysis indicated that 100 % of the cell aggregates from the fermentor were <0.25 mm^2, and their size distribution remained constant during cultivation. The cultures grown in shake flasks presented a tendency to increase their aggregate size as a consequence of cell growth. The reduction of the size of *S. chrysotrichum* aggregates during the scale-up had an important influence on flow index behavior and consistency coefficient values.

Key words: *Solanum chrysotrichum*, plant cell morphology, broth rheology

Introduction

Plant cell culture has been receiving attention as an alternative for the production of valuable plant-derived secondary metabolites, because of its many advantages over whole plant cultivation. However, more research is required to enhance culture productivity and to reduce processing costs, which will be the key to the commercialization of plant cell culture processes (*1*,*2*).

The rheology of plant cell broths is an important factor for the bioprocess development, because it determines the bioreactor power requirement and mass transfer characteristics (*3*). However, there are few reports on this subject. Doran (*3*) and Sánchez *et al*. (*4*) published a summary of relevant studies related to the rheology of plant cell suspension cultures. These authors mentioned that the majority of suspension cultures show non-Newtonian characteristics. The broths showed pseudoplastic behavior, which was associated with biomass concentration and cell morphology (*5–8*). Plant cell cultures are

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normally composed of a complex mixture of single cells and aggregates with different shapes and sizes (*9,10*). Curtis and Emery (*7*) demonstrate that morphological characteristics of the cell population determine the rheological behavior of the broth. Single cells and aggregates of the same culture can usually be observed under a microscope; however, their count or measure is a laborious and difficult task. Reports indicate that in a sample of the same culture it is possible to find cells with a diameter varying between 10 and 120 μm, whereas cell aggregates can have a size of several millimeters with round or elongated morphology (*1,11*). Alternatively, image analysis may be considered as a powerful tool to measure the microscopic characteristics of plant cells (*12*). Recently, this technique was used to obtain data on morphology of suspension cultures of *Oryza sativa* (*13*), *Nicotiana benthamiana* (*13*), *Trichosanthes kirilowii* (*13*), and *Solanum chrysotrichum* (*9,10*), and to evaluate the pigment production in cell cultures of *Fragaria ananassa* (*14*).

Solanum chrysotrichum (called »Sosa«), a Mexican species of Solanaceae, is one of the most frequently used plants in the southeastern region of Mexico for the treatment of various skin mycoses such as *Tinea pedis* (*15*). *S. chrysotrichum* produces saponin (SC-1), which is used as an antifungal agent in treating skin mycosis. Plant cell tissue culture of this species was established by Villarreal *et al.* (*16*). Considering that cell morphology and aggregate size may be affected during the scale-up of the cultures, the aim of this work was to compare the kinetic profile and cell morphology of *S. chrysotrichum* cultivated in shake flasks and in a 2-L stirred tank fermentor and to analyze their influence on the flow behavior of the broths.

Materials and Methods

Cell cultures

Cell suspension cultures of *S. chrysotrichum* were obtained according to the methodology reported by Villarreal *et al.* (*16*). Cells were grown in Murashige and Skoog (*17*) medium, supplemented with sucrose (30 g/L), 2,4-dichlorophenoxyacetic acid (2 mg/L) and kinetin (2 mg/L). The medium pH was adjusted to 5.7 prior to sterilization.

Shake flask culture

S. chrysotrichum culture was carried out in 125-mL Erlenmeyer shake flasks with 30 mL of culture medium with an inoculum of 1.0 g of fresh weight (FW). The flasks were shaken at 100 rpm, with continuous illumination of 40 μmol/(m^2 s) and (25±2) °C. Two flasks were removed for analytical measurements every 3 days. The results reported are the average of 3 independent experiments.

Bioreactor culture

A 2-L fermentor (Applikon, Schiedam, The Netherlands) with a jacketed glass vessel and a multiport, stainless steel head plate was used. Configuration of the fermentor and the operation conditions were the same as reported by Rodríguez-Monroy and Galindo (*6*). One turbine impeller (4.6 cm) with four blades (1.1 cm) operating at agitation speed of 400 rpm was used. The impeller was positioned 2.6 cm above the bottom of the vessel. The tank diameter was 13 cm. Aeration (0.1 v.v.m.) was provided via a ring sparger positioned below the turbine. Temperature was maintained at (26±2) °C. The fermentation vessel (containing 1.5 L) was inoculated with 150 mL of 7-day-old suspension culture grown in a 500-mL shake flask. A sample of 30 mL was removed from the vessel for analysis every 3 days.

Analytical methods

Biomass

Fresh weight mass (FWM) was determined by filtration of 3-mL aliquot through a filter paper of known dry weight mass. The cells were dried to constant weight (70 °C, 1 day) in order to obtain the dry weight mass (DWM).

Apparent viscosity

Measurement was made according to Sánchez *et al.* (*4*). The viscosity of the whole broth and the filtrate was measured using a Haake viscometer (Rotovisco RV20, Germany), equipped with a double gap system (NV, 0.35 mm gap width). The range of shear rate was from 27 to 2700 s^{-1} at 25 °C. The rheological behavior was adjusted to the Ostwald de Waele model (power law):

$$\tau = k\dot{\gamma}^n$$

where τ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), k is the consistency coefficient (Pa s^n) and n is the flow behavior index (dimensionless). In order to determine the values of n and k, a plot of log τ *vs.* log $\dot{\gamma}$ was obtained; the slope of the line represents n and the intercept represents log k.

Image analysis

The technique reported by Trejo-Tapia *et al.* (*10*) was used. Images of the cells were obtained using a microscope (Nikon, Alphaphot-2 YS2, Tokyo, Japan) with a charge-coupled device camera (Nikon, Coolpix 900, Tokyo, Japan). Magnification of the image was 4×. The image analysis software was Meta Imaging series for Microsoft Windows (version 4.0, Universal Imaging Corporation, Downingtown, Pennsylvania, USA), in which basic image processing tools were used to count aggregates and to measure their area (mm^2), length (mm), breadth (mm) and elliptical form factor (EFF). EFF was calculated as the length/breadth ratio, where an EFF of 1 represents a round aggregate, whereas an EFF >1 indicates an elongated aggregate. For each sample, the morphology of 200 aggregates was measured. Standard errors of image analysis were <0.5 %.

Results

Fig. 1 shows that the growth curves of *S. chrysotrichum* cells cultivated in a shake flask and in a stirred tank had a similar profile until day 14. The cultures presented a specific growth rate of 0.09 day^{-1}, and the FWM biomass concentration obtained on that day was 130 g/L. This value corresponds to DWM concentration of 10 g/L. After 14 days, the cultures developed in the fermentor

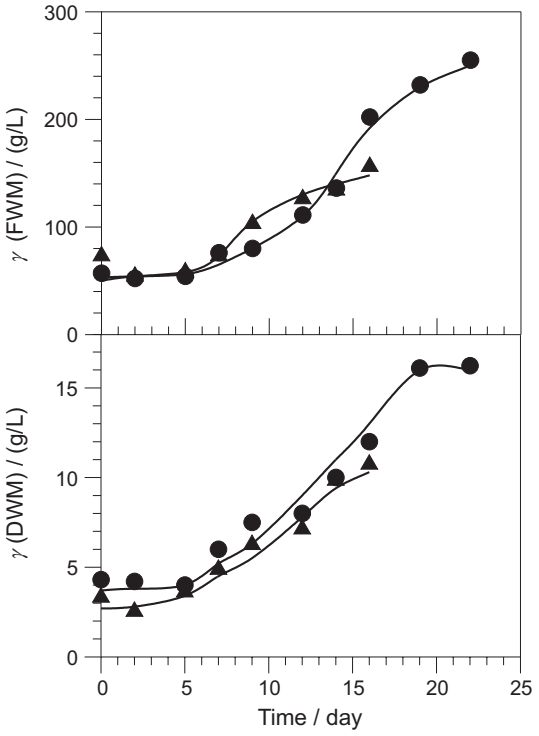

Fig. 1. Kinetics of *S. chrysotrichum* cultures grown in shake flasks (●) and in a stirred tank fermentor (▲)

reached a maximum FWM biomass concentration of 157 g/L (DWM concentration of 10.7 g/L) at day 16. The cultures performed in the shake flask presented a significant increase in its fresh weight, with a maximum FWM biomass concentration of 250 g/L (DWM concentration of 16 g/L) at day 22.

In order to evaluate the possible effects of mixing conditions on the morphological properties of the cultures, a comparative analysis of *S. chrysotrichum* aggregate size was made in samples obtained from shake flasks and fermentor at the same fresh biomass concentration (Fig. 2 and Table 1). At the beginning of the culture (FWM concentration of 50 g/L) the aggregate size profile was similar in both systems, with particle size of <0.25 mm^2 and average aggregate area of 0.059–0.068 mm^2. The aggregates obtained in the shake flasks increased their size during growth, and reached an average aggregate area of 0.160 mm^2 at FWM concentration of 200 g/L, while the aggregate size profile of the cultures developed in the fermentor was constant for all biomass concentrations evaluated (average cell aggregate area of 0.05 mm^2). Cell aggregates obtained in the shake flask (at the same concentration) were always larger than those obtained in the fermentor and a maximum difference between both systems was observed in the stationary phase.

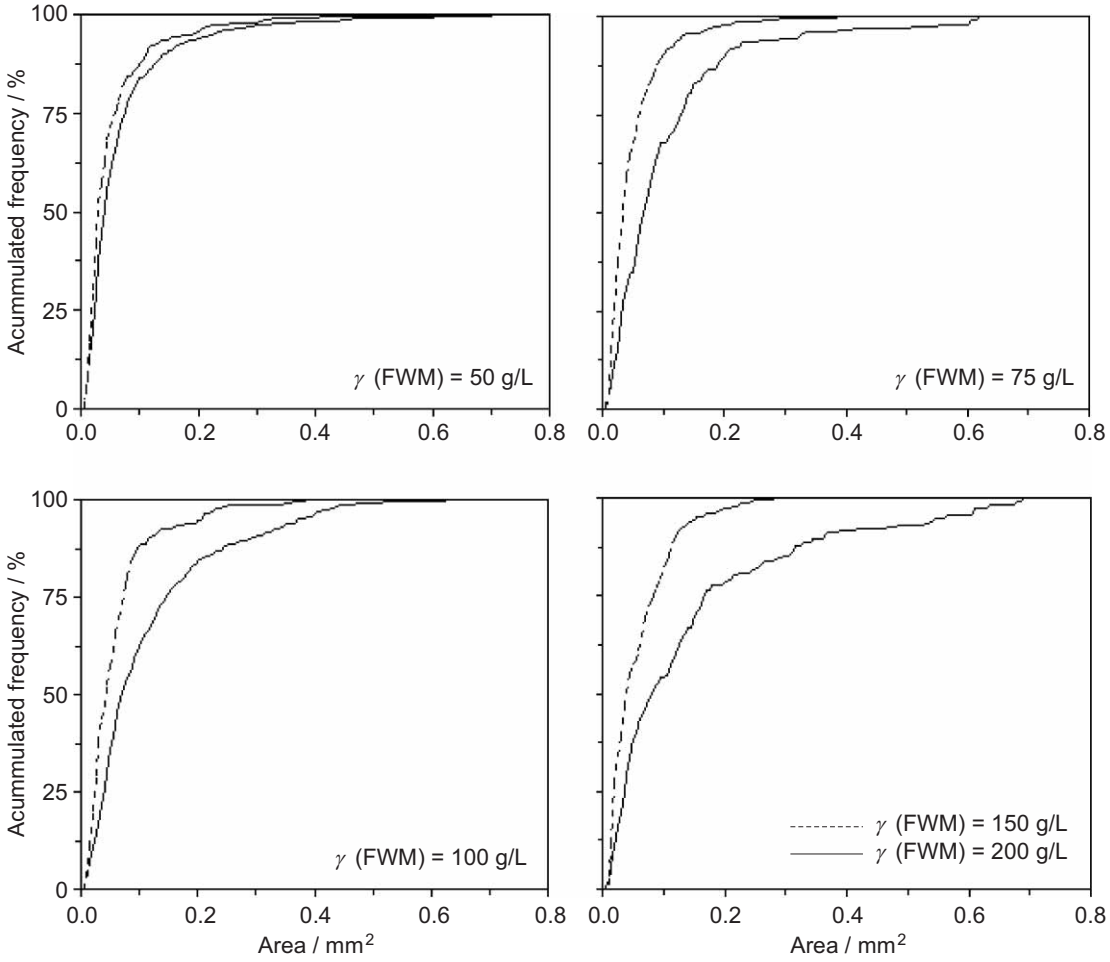

Fig. 2. Accumulated frequency of aggregate size of *S. chrysotrichum* cultivated in shake flasks (——) and in a stirred tank fermentor (-------)

Table 1. Average size of cell aggregates of *S. chrysotrichum* suspension cultures developed in shake flasks and in a stirred tank

γ(FWM) g/L	Cell aggregates area mm^2	
	Shake flasks	Stirred tank
50	0.068 ± 0.009	0.059 ± 0.0006
75	0.112 ± 0.005*	0.054 ± 0.014
100	0.111 ± 0.010*	0.059 ± 0.006
150	n.d.	0.058 ± 0.007
200	0.160 ± 0.022*	n.d.

n.d. not determined
*$p \leq 0.05$

EFF was used to characterize the aggregate roundness. The results in Fig. 3 show that *S. chrysotrichum* cultures developed in a shake flask as well as in a fermentor had values of EFF between 1 and 5, showing very similar profiles. The FWM concentrations of 50 and 125 g/L were used as typically low and high biomass concentrations, respectively, showing that in both cultures EFF was not dependent on the biomass concentration.

In order to determine the effects of the difference in aggregate size on the flow behavior of *S. chrysotrichum* broths, the rheograms at the same biomass concentration were compared (Fig. 4). The results show that the

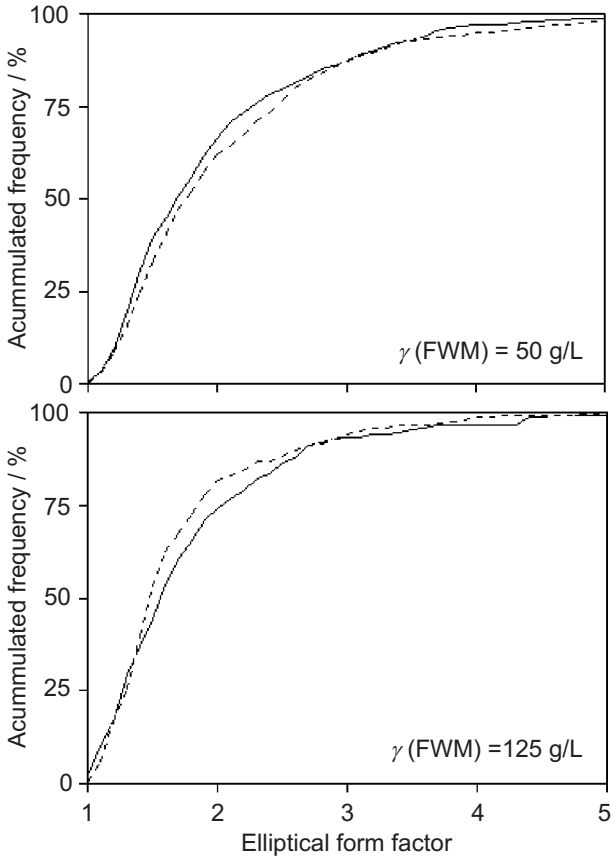

Fig. 3. Accumulated frequency of aggregates as a function of the elliptical form factor of *S. chrysotrichum* cultures developed in shake flasks (——) and in a stirred tank fermentor (-------)

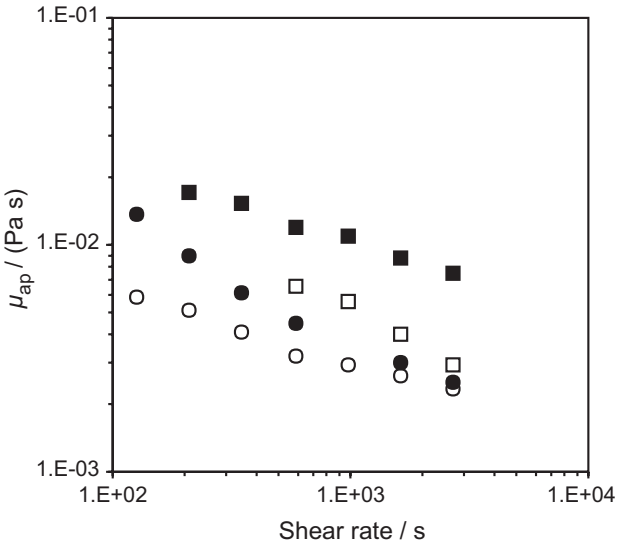

Fig. 4. Rheograms of *S. chrysotrichum* broths developed in shake flasks (□,■) and in a stirred tank fermentor (○,●) at different FWM biomass concentrations (○,□ 50 and ●,■ 75 g/L)

culture broths obtained from shake flasks as well as from the fermentor exhibited a non-Newtonian rheological behavior, which was characterized as pseudoplastic. Pseudoplasticity of the broths was caused by the biomass concentration (cell-free medium was Newtonian with viscosity close to the value of water, data not shown). Flow behavior index (n) and consistency coefficient (k) of the power law model were obtained to determine the effects of different aggregate size population (Table 2). Considering the results obtained in the fermentor, where the profiles of aggregate size and EFF were similar for all samples, it was possible to analyze the effects of the biomass concentration on the flow behavior index and consistency coefficient. An increase in FW biomass concentration from 50 to 75 g/L caused the increase of the consistency coefficient 3.9 times; while the flow behavior index decreased from 0.67 to 0.54. A different relationship was observed in the rheological properties of the cultures developed in shake flasks. In this case, the consistency coefficient was 7.1-fold with respect to the initial value, as a consequence of the increase of biomass, while the flow index increased from 0.77 to 0.85. These results indicated that the morphological properties of the aggregates strongly determine the rheological behavior of the culture.

Table 2. Flow behavior index (n) and consistency index (k) of the power law model for cultures of *S. chrysotrichum* obtained in shake flasks and in a fermentor

System	γ (FWM biomass) g/L	Flow index (dimensionless)	Behavior consistency index $mPa\ s^n$
Fermentor	50	0.67	28.2
	75	0.54	111.9
Shake flasks	50	0.77	13.18
	75	0.85	99.21

Discussion

The results obtained in this work show that the biomass yield of *S. chrysotrichum* cultures developed in a fermentor was 60 % lower than that obtained in a shake flask (Fig. 1). The dissolved oxygen tension of the cultures growing in the fermentor was maintained above 10 % to safeguard a possible oxygen limitation (data not shown); in consequence the low biomass yield obtained in the fermentor by an oxygen limitation is unlikely, as indicated by the comparison with the rate of growth in shake flasks. It is possible that the mixing conditions used to grow *S. chrysotrichum* in the stirred tank, which generated higher shear rates, could negatively affect the obtained growth yield. In this respect, the results obtained in this work are consistent with the negative effects reported for other species during the scale-up from a shake flask to the fermentor, for example *Beta vulgaris* (*6,18*) and *Nicotiana tabacum* (*19*). It is necessary to indicate that fluid mixing is an important factor in the process optimization and scale-up of suspension cultures for the efficient production of useful metabolites. This was demonstrated with *Taxus chinensis* for taxoid production (*20*).

Separation of plant cell population using a series of standard sieves has been used as an approximation to describe the aggregate size distribution, considering that the fresh or dry weight retained in each one of the sieves represents a percentage of the total population (*11*). Recently, it was demonstrated that image analysis is a more comprehensive alternative to describe the morphological properties of *S. chrysotricum* cultures (*10*). The results obtained in this report demonstrated that the morphological characteristics of *S. chrysotrichum* cells were influenced by the system used for growing cultures (shake flask and stirred tank). The cultures carried out in shake flasks showed a clear tendency to increase the aggregate size during growth (Fig. 2), while the aggregate size of cultures in a fermentor remains constant. However, the shapes of the aggregates represented by the elliptical form factor observed in both populations were very similar, regardless of the biomass concentration. There are reports indicating that plant cell cultures present a tendency to grow forming aggregates (*1*); however, the results presented in this work demonstrate that the size of *S. chrysotrichum* aggregates is determined by the mixing conditions employed in growing the cultures (shake flask or fermentor). These results could be in opposition to those reported for *B. vulgaris* (*6*). That work compared the aggregate size of a culture developed in a shake flask as well as in the fermentor using a particle counter (Coulter). The results indicated that *B. vulgaris* aggregates were similar in both systems. Morphological size could be a cause of difference between *S. chrysotrichum* and *B. vulgaris*. *S. chrysotrichum* aggregates in shake flasks were up to 0.4 mm in diameter, while the aggregates of *B. vulgaris* reached only 0.1 mm. Furthermore, the analysis with the particle counter is limited in comparison with image analysis, because of the assumption that the entire aggregate has a spherical shape.

It is possible that the small size of *S. chrysotrichum* aggregates observed in the fermentor is a consequence of the physical limitations of growth imposed by the hydrodynamic stress conditions in the fermentor in contrast with the culture developed in shake flasks (a system considered to have low stress conditions) where the cells showed a tendency to form large aggregates with sizes up to 0.5 mm^2.

S. chrysotrichum cells were responsible for the pseudoplastic behavior of the broths because the filtered medium of *S. chrysotrichum* had a Newtonian behavior with a viscosity of 1 cP. It is in agreement with a previous report on cell culture of *Perilla frutescens* (*8*). However, these results do not agree with those reported for *B. vulgaris* (*4,6*) and *Nicotiana tabacum* (*21*). In both species, the pseudoplastic behavior of the broths was determined by the biomass concentration as well as the extracellular compounds produced by the cells, as a possible response to high hydrodynamic stress present in the fermentors (*6,11*). In this work, the operational conditions used to grow *S. chrysotrichum* cultures were the same as those used for *B. vulgaris* cells (*6*). Therefore, it is possible that the accumulation of the extracellular compounds, observed previously by other cultures (*4,6,21*) could be a particular property of plant species and a phenomenon not well understood. There is a hypothesis that the incorporation of viscous compounds in the medium may generate a protector effect for fragile systems, such as insect (*22*), algal (*23*), and somatic embryogenic cultures (*24*). In this context, the culture of *S. chrysotrichum* utilized in this work could be considered as a resistant species to the growth in a stirred tank without the addition of a protective substance or production of a viscous substance.

Fig. 4 and Table 1 show that the broths of *S. chrysotrichum* exhibited a non-Newtonian rheological behavior, which was characterized as pseudoplastic. This behavior was independent from the system used for growing the cultures. However, the sizes of the aggregates generated in shake flasks were higher than those obtained in the fermentor, these properties made differences in the values of flow behavior index and consistency coefficients. It is also reported that cell/aggregate morphology was very important for rheological properties of plant cells (*8*).

Conclusion

S. chrysotrichum broths developed in shake flasks as well as the broths obtained in a stirred tank bioreactor exhibited non-Newtonian and shear-thinning characteristics. Pseudoplasticity of the broth was determined by the biomass concentration and by the aggregate size properties of the culture. The results of morphological analysis indicated that *S. chrysotrichum* cultures in a stirred tank fermentor presented aggregate sizes smaller than those in a shake flask. However, the aggregate roundness remains constant in both systems. The changes in the size distribution determine that the pseudoplasticity of the broths obtained in each system was different.

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Utvrđivanje morfoloških svojstava stanične kulture *Solanum chrysotrichum* na tresilici i u fermentoru te reoloških svojstava podloge

Sažetak

Stanice *Solanum chrysotrichum* uzgajane su na tresilici i u bioreaktoru s miješalicom. Određivani su kinetika rasta i morfološka svojstva stanica te reološka svojstva podloge. Specifična brzina rasta bila je slična u oba sustava (0,09 dan^{-1}), dok je povećanje biomase na tresilici bilo 60 % više nego u fermentoru. Podloge za uzgoj pokazivale su nenjutnovske značajke te osobine popuštanja smičnog naprezanja koje se mogu simulirati modelom Ostwald de Waelea. Rezultati pokazuju da pseudoplastičnost podloge za uzgoj ovisi o koncentraciji biomase i raspodjeli veličine agregata, dok je zaobljenost agregata, mjerena eliptičnim faktorom oblika, bila slična u oba sustava. Morfološka analiza pokazuje da je 100 % staničnih agregata iz fermentora veličine <0,25 mm^2, a njihov je način razdiobe u podlozi ostao konstantan tijekom uzgoja. Kulture uzgojene na tresilici imaju tendenciju povećanja veličine agregata, što je posljedica rasta stanica. Prelaskom na uzgoj u fermentoru bitno se smanjuje veličina agregata *S. chrysotrichum*, što utječe na indeks protjecanja i vrijednosti koeficijenta gustoće.