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Submerged and Solid-State Phytase Fermentation by *Aspergillus niger*: Effects of Agitation and Medium Viscosity on Phytase Production, Fungal Morphology and Inoculum Performance

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Summary

Qualitative relationships between agitation levels and medium viscosity, *Aspergillus niger* morphology and phytase production were investigated in submerged and solid-state fermentations. Overall phytase production increased with increasing shaker speeds from 150 to 300 rpm, although specific growth rates and phytase production rates were higher at 150 rpm for 72 h from inoculation. Fungal morphology was greatly influenced by agitation with the morphological forms of small pellets and entangled mycelia predominating at 150 rpm, while the free filamentous form was obtained at 300 rpm. Upon inoculation of SSF, increased productivities were obtained from inocula grown at 150 rpm. A shift towards the filamentous growth form was observed when guar gum was added to the liquid media, which increased the viscosity from 2 000 cp (medium without gum addition) to 52 000 cp (addition of 1 g/L gum). At both shaker speeds, with the effect being more pronounced at 300 rpm, phytase production increased with gum addition. Specific growth rates for the first 72 hours from inoculation, were higher at 150 rpm, irrespective of gum concentration, while solid-state fermentations inoculated with these cultures led to higher amounts of phytase compared to those obtained from 300 rpm inocula.

Key words: phytase, *Aspergillus niger*, solid-state fermentation, inoculum, fungal morphology, agitation, viscosity

Introduction

The use of filamentous fungi for the production of commercially important metabolites has increased rapidly over the past half century and the production of enzymes in submerged fermentation (SmF) has long been established. In recent years research interest in batch solid-state fermentation (SSF) has addressed the production of many innovative and high value products, *e.g.* single-cell protein (SCP), protein enriched feed, ethanol, enzymes, mycotoxins, from starchy materials and a variety of wastes utilized by fungi (*1*). In general, SSF is

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simple, in terms of equipment and process control, less expensive and often results in higher yields compared to submerged fermentation (*2*).

Fungal cultures adopt different growth patterns when cultivated in liquid and solid substrates. Under SmF conditions, they are exposed to hydrodynamic forces, while in solid-state systems growth is restricted to the surface of the solid matrix. Research studies on fungal growth patterns in solid-state cultures are limited; while the development of fungal morphology in SmF is well investigated for a number of industrially important fermentations (*3–6).* In liquid environments, fungi grow as pellets or free mycelia, depending on the genotype of the strain and culture conditions. Each form has its own characteristics which greatly affect the process yields (*3*) and attempts have been made to manipulate morphology in order to achieve increased productivity (*7).*

Among the factors which influence process yields, the quality of the inoculum is regarded as the parameter that determines the course of a fungal fermentation (*8*). Inoculum morphology has been studied and correlated with process yields for a number of important submerged fermentation processes (*9–11*). On the other hand, published research concerning morphological aspects of inoculum for solid-state fermentations is very limited. In an earlier publication (*11)* we presented results that outlined the relationship between the vegetative inoculum characteristics and phytase production in SSF.

Phytase is the enzyme that releases inorganic phosphates from phytic acid. Phytic acid is the natural organic form of phosphorus which is not readily assimilated by animals, binds essential dietary minerals, reducing this way their availability for absorption (*12*), and moreover inhibits a number of nutritionally important enzymes *in vivo* (*13).* Phytase can be produced by many species of fungi and bacteria (*14*), while strains of *Aspergillus niger* produce large amounts of the enzyme in solid state fermentation (*15*).

Spores production was not effective for the strain used, therefore a liquid preculture was needed to provide a vegetative inoculum for SSF. In that work, phytase production and *Aspergillus niger* growth and morphology were studied in both SmF and SSF and medium composition (two medium formulations used) effects were investigated. The characteristics of the submerged fermentations were evaluated independently and also in terms of its suitability for use as inocula for solid-state fermentations. Fungal morphology was the parameter that determined phytase production levels in both SmF and SSF. In the present paper further investigation of phytase production and growth characteristics in that particular system was done. In the first part of the work (published *(11)*) two media formulations (two dilution levels of the same ingredients) were compared, and a significant difference in fungal morphology and phytase production between the two was found. It is interesting to investigate whether using one medium formulation, viscosity variations, by means of gum addition, as well as shaker speed variations in the inoculation culture influence its performance as inoculum for SSFs.

Materials and Methods

Microorganism

A phytase-producing strain of *Aspergillus niger* (provided by Altech Inc.) was used throughout this work. Culture was maintained by means of monthly subculture on PDA agar and storage at room temperature. Inoculation was done by cutting an agar block (5 x 5 mm) from the growing edge of the culture and transferring it to the centre of the new plate.

Inoculation was performed using an agar block of 5 x 5 mm from the agar plate to inoculate 100 mL of medium in a 250 mL shake flask.

Submerged Fermentation

The standard semi-synthetic fermentation medium (M1 medium) used in this work, contained in g/L: cornstarch, 28; glucose, 5; peptone, 18; KCl, 0.5; $MgSO_4 \cdot 7H_20$, 1.5; KH_2PO_4, 1; $CaCl_2 \cdot 2H_20$, 2. Media, 100 mL in 250 mL Erlenmeyer flasks, were sterilized at 120 °C for 20 min (autoclave pressure: 0.14 MPa). Sterile wheat bran was added to the sterile medium prior to inoculation. The pH after sterilization was between 5.0–5.3. When guar gum was included, it was added to the medium prior to sterilization. Viscosity was measured afterwards with a portable viscometer (Helipath with T-Bar Spindles) at 30 °C which was the fermentation temperature. Fermentations were carried out on an orbital shaker incubator (New Brunswick Scientific).

Solid-state Fermentation

Solid-state fermentations were carried out in 250 mL Erlenmeyer flasks, in controlled temperature chambers, at 30 °C. The solid substrate contained in g per flask: wheat bran 3.5 and full-fat soy bean meal 1.5. Sterilization was done at 120 °C for 20 min (0.14 MPa autoclave pressure). The moisture content was adjusted by addition of sterile water prior to inoculation to 53 %. Three mL of the liquid shake flask culture was used to inoculate the solid-substrate flask. 72-h old liquid shake flask cultures were used as inocula for all solid-state fermentations.

Analytical Methods

Phytase activity was determined in the filtrate of shake flask cultures for the SmF and following extraction from the fermented bran for the SSF. The method used and the procedure followed for SmF and SSF runs were as described in an earlier publication (*11*).

Fungal biomass measurements in SmF were made by filtering 20-mL liquid samples through dried and pre-weighed glass microfilters. The exact procedure was described in an earlier publication (*11*). In SSF biomass levels were determined by measuring the glucosamine concentration of the mycelium with the Erlich's reagent using the method of Shakurai *et al.* (*16*). The glucosamine content of cells was expressed as mg glucosamine per gram of fermented solid mass containing both substrate and cells.

Pellet diameters were measured using a graduated microscope slide. Measurements were made on 144

hours samples. The average diameter of 40–50 pellets per sample was calculated and presented in this work.

Experimental Design and Data Analysis

Two levels of shaker speed were examined, 150 and 300 rpm, for submerged fermentation and subsequent inoculum production. Three levels of gum concentration were evaluated within each shaker speed, 0, 0.5 and 1 g/L. The experimental design was a split-plot, with shaker speed representing the whole plot, and gum concentration being the treatment within the whole plot. Twelve flasks for each gum concentration treatment were used to allow for two sub-samples (averaged to determine the replicate value) at each of the six sampling times (24, 48, 72, 96, 120 and 144 h) in SmF. The flasks were sampled without replacement. Experimental design was identical for the SSF inoculum. 72 h inoculum was used for SSFs which were incubated at 30 °C for 5 fermentation lengths. Experiments were replicated twice.

Data were analysed with SAS using PROC GML. The data were blocked on replication to remove possible effects of differences in original inoculating cultures from one experimental replication to the other. Shaker speed was analysed as the whole plot, with the Experiment / Shaker speed interaction term used as the whole plot error term. Gum concentrations and the shaker speed by gum interactions were tested for significance using the mean square error of the analysis of variance.

Results and Discussion

The factors that influence the morphology of filamentous fungi in submerged culture are numerous. Mechanical parameters, which are simply referred to as agitation (*7*,*17*), the composition of the culture medium (*18*,*19*), as well as the viscosity of the medium (*20*) are some of the parameters that influence morphology and process productivity. In an earlier publication we presented work on the effect of medium composition on phytase fermentation (*11*). Using two medium formulations, the standard, M1, as described in the previous section and a modified formulation with a ten-fold lower concentration of cornstarch, glucose and peptone, medium M2, with concentrations of cornstarch, glucose and peptone of 2.8, 0.5 and 1.8 g/L, respectively. Both media were tested with and without wheat bran supplementation. We observed a significant difference in the development of the morphology of microorganism and phytase production levels between the media. Fungal morphology was found to be related to phytase production. Small pellets and filamentous mycelia resulted in higher phytase activities than large pellets and furthermore inoculation of solid-state fermentations with a mixture of small pellets and filamentous mycelia resulted in all cases in increased phytase activities.

Different starch concentration in the two media formulations led to different medium viscosities. Apart from the influence on mass transfer, viscosity has been found to be related to fungal morphology in certain fermentations (*20*,*21*). It would be interesting therefore to see, keeping one standard medium formulation and changing only the initial viscosity, what the relationship is between phytase production and viscosity in submerged culture, and in solid-state when such a culture is used as inoculum for SSF. The other factor examined in the present work is the effect of agitation, in terms of shaker speed, on phytase production in SmF and the performance of the inoculum cultures grown at different agitation levels in subsequent SSF. As described in the Materials and Methods section, experimental data were analysed to determine the significance of the parameters and possible gum/speed interactions, over the time-course of fermentations.

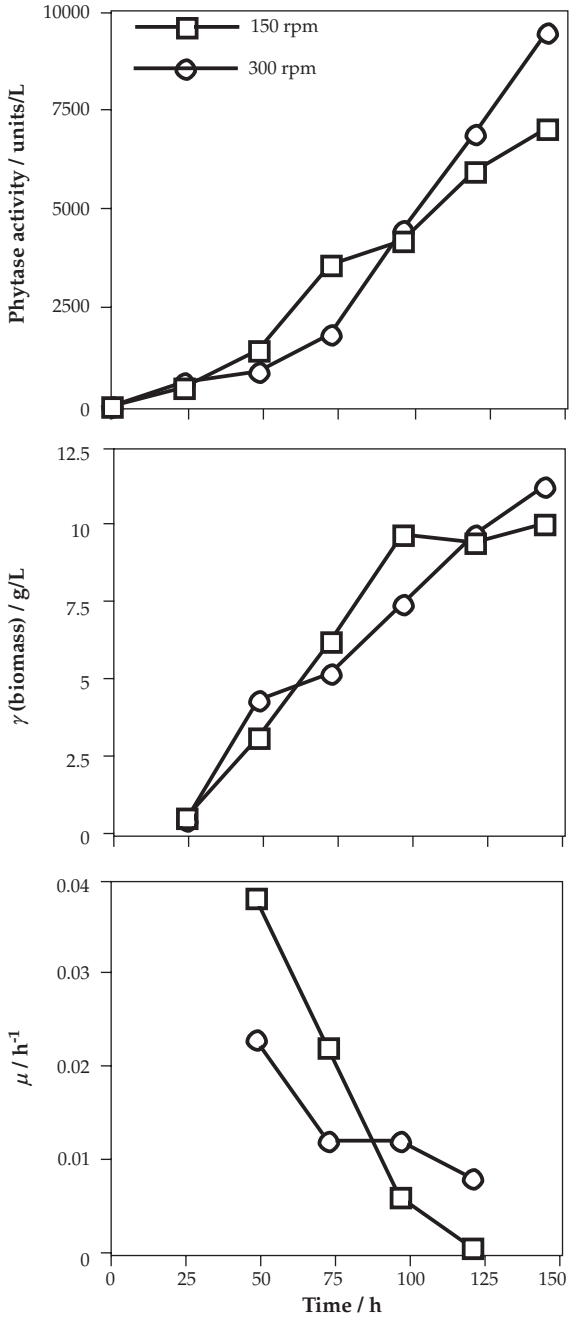

Fig. 1. Time courses of phytase activities, biomass concentrations and specific growth rates in submerged fermentation at 150 and 300 rpm

The effect of shaker speed – Submerged Fermentations

Comparing the two shaker speeds, overall phytase activity was higher at 300 rpm at 150 hours (Fig.1). Phytase levels at 150 rpm were initially equal to or greater than the 300 rpm treatment, until 120 hours, when the 300 rpm treatment surpassed the 150 rpm. Biomass levels were essentially equivalent between the two treatments, although it appears that growth leveled off the 150 rpm treatment at 96 hours. As shown in Fig. 1, the decrease in specific growth rate was much larger for 150 rpm than for 300 rpm. The calculated specific growth rates between 48 and 72 hours were 0.022 h^{-1} at 150 rpm, while 0.012 h^{-1} at 300 rpm. This will be important when we discuss the solid-state fermentation results.

Light microscopy of broth samples from the treatments at 150 rpm, revealed a mixture consisting mainly of fine pellets of an average diameter of 0.5 mm and a small number of entangled mycelia (clumps). In the 300 rpm treatment, no pellets were detected and the mycelium was in the form of loose clumps and free (non aggregated) mycelial trees.

Factors that influence fungal morphology in submerged culture have been discussed in many cases. Concerning the pelleted form, investigations have shown factors which favor cell aggregation and promote pellet formation, while others favor dispersion between microorganisms resulting in filamentous forms (*21*). The shear stress exerted in a fungal culture, in the form of hydrodynamic forces due to agitation, is regarded as one factor that favors dispersion and this indeed would be the case in the present work: increased shaker speeds discouraged pellet formation.

A direct relationship between agitation and phytase production cannot be drawn from the results, unless we take into account the fungal morphology in the particular fermentation. From the trends of the phytase time courses we cannot simply conclude that increased agitation favors phytase production by the fungus. It is very likely that fungal morphology played a key role in the process. The two different agitation levels resulted in the development of distinctive morphological forms. Fine pellets gave increased phytase levels up to 90 hours and this morphology was associated with increased specific growth rates for the first 72 hours of fermentation. Following this time, growth and phytase production rates decreased in the 150 rpm treatment. This was not the case with the morphological form obtained at 300 rpm, the filamentous mycelium.

Formation of mycelial pellets is often regarded as a prerequisite for successful production of microbial metabolites, including some fungal enzymes such as polygalacturonidase or glucosidase (*22*). In other cases, the free filamentous morphology is preferred for optimal metabolite formation (*18*). It has been shown that differentiation of mycelia during pellet formation results in striking effects on enzyme production. Polygalacturonidase synthesis correlates well with the pelleted morphological type of *A. niger*. The more compact the pellet, the greater the polygalacturonidase synthesis, regardless the composition of the medium (*22*). Such phenomena, usually related to diffusional limitations in pellets and catabolic repression, suggest that pellets may be regarded as complex differentiated tissues and not merely as mechanical conglomerates (*21*). In our case, the increased rates of phytase production from pellets may suggest that this morphological form is more suitable for the phytase fermentation when it extends for about 90 hours from inoculation. However, as studies have shown (*23,24*) the center of the pellets becomes autolysed with time and does not contribute to metabolite biosynthesis anymore. In other words, a lower proportion of the biomass is involved in biosynthesis and this can explain the reversing trend observed when passing 90 hours of fermentation. The results obtained suggest that the extent of pellet autolysis is small, which is expected, provided nutrients are available, since in general center autolysis is more profound in pellets of much larger diameters (*24*). In the filamentous growth form a lower proportion of cells becomes autolysed with time and this can result in increased final productivities compared with the pelleted growth form.

The effect of shaker speed – Solid-State Fermentations

72-h old shake flask cultures were used as inocula for the solid-substrate fermentations. *Aspergillus* growth and phytase production were much higher when the inoculum from the 150 rpm SmF was used (Fig. 2). Phytase activities at the end of SSF runs (144 h) were 64 % higher compared to those obtained from SSF inoculated with the 300 rpm precultures. Growth in SSF was evalu-

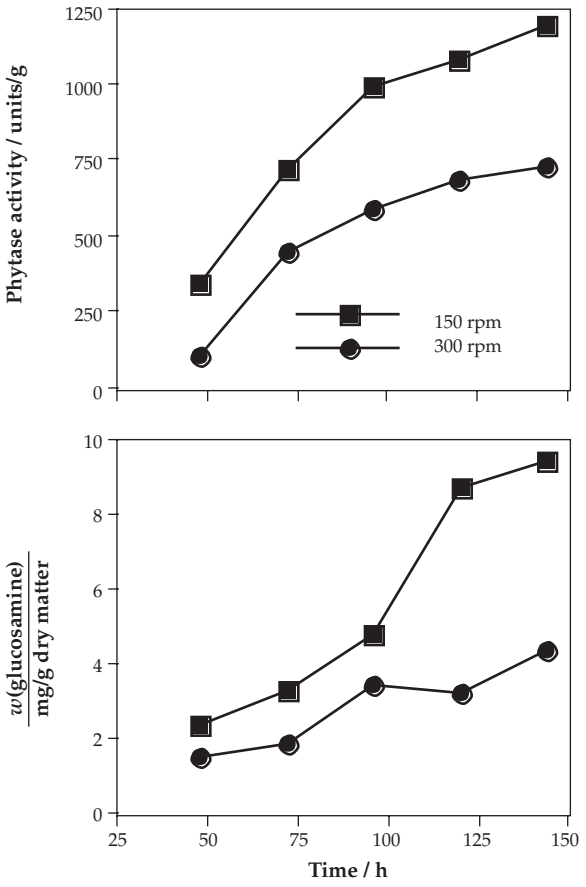

Fig. 2. Time courses of phytase activities and glucosamine concentrations in solid-state fermentation inoculated with cultures grown at 150 and 300 rpm

ated by the estimated glucosamine content of the mycelium per gram fermented mass over time. Glucosamine content was significantly higher in the case of the 150 rpm – grown inoculum. Phytase production in SSF was growth related.

Comparing the characteristics of the two inoculum cultures used for SSF, we observe that the 150 rpm inoculum had only a slighly higher initial biomass concentration compared to that of the 300 rpm inoculum, so that this could not be solely responsible for the SSF outcome. However, the specific growth rates of the two cultures at the particular time of 72 h were significantly different: 0.012 h^{-1} for the 300 rpm and 0.022 h^{-1}, almost double, for the 150 rpm culture. This is most likely a function of the morphological profile of the cultures. The mycellium in the form of fine pellets and increased specific growth rates made a more suitable inoculum than the filamentous form which was in a state of lower specific growth rates.

As we discussed in the beginning of this section, in an earlier publication on phytase fermentation which was based on the same system, the morphology of the fungus responded significantly to changes in medium composition, by taking distinctively different forms. Each form resulted in different productivities when used as inoculum in SSF. The results presented in this paper further support the importance of fungal morphology as an inoculum parameter which determines the SSF outcome, irregardless of how the morphological form was obtained.

The two inoculum cultures also contained very different levels of phytase: the 150 rpm inoculum contained 96 % more phytase than the inoculum grown at 300 rpm. This may mean that phytase in the inoculum may act as an inducer for increased productivities in SSF. However, the role of phytase in the inoculum cannot be confirmed from these results and further investigation is necessary.

The effect of medium viscosity – Submerged Fermentation

The initial viscosities of the medium with 0, 0.5 and 1 g/L gum, were 2 000, 27 750 and 52 000 cp, respectively. These numbers were calculated from readings taken with identical scales and rotation, and using the same T-A spindle.

Comparing the runs performed at varying viscosities, we observed that final phytase activities increased with increasing gum concentrations (Fig. 3).

Data analysis showed that there were no treatment differences until the 72nd hour sampling point. At 72 h, gum concentration was statistically significant, as was the shaker speed/gum interaction (P=0.05). At 300 rpm, increased gum concentration resulted in more phytase and this effect was more pronounced than at 150 rpm. At 96 h, gum concentration was significant (linear effect). As gum concentration increased, phytase production also increased over all speeds. At 120 h, the parameter shaker speed was significant: 300 rpm cultures produced 11 % more phytase than 150 rpm cultures, averaged over all gum concentrations. Gum concentration was also significant, since phytase increased linearly with gum concentration. At 144 hours, shaker speed was significant and 300 rpm cultures produced 47 % more phytase than 150 rpm cultures. At this sampling point there was some indication of speed/gum interaction, with 300 rpm having a stronger linear response to gum concentration than the 150 rpm cultures.

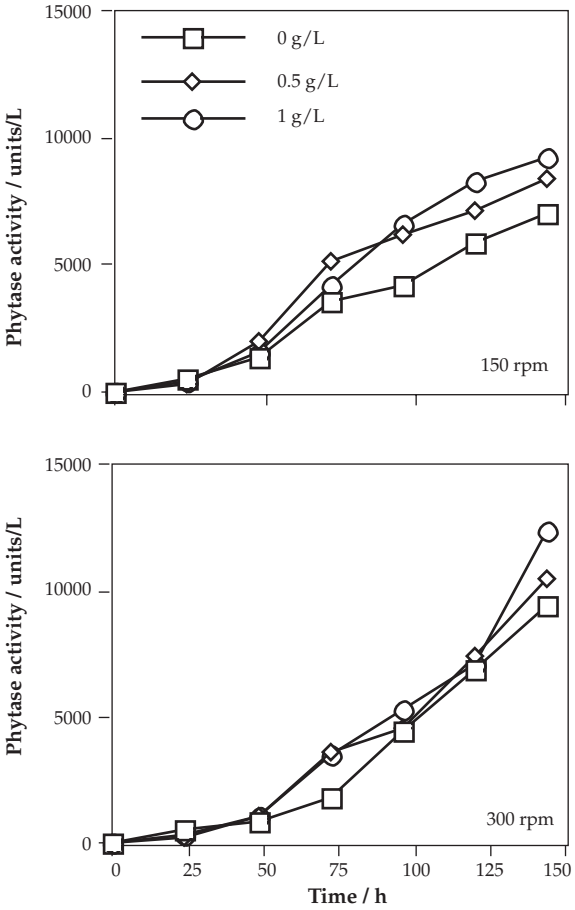

Fig. 3. Time courses of phytase activities in submerged fermentation at 150 and 300 rpm and gum concentrations at 0, 0.5 and 1 g/L

As was observed comparing the shaker speeds, final biomass levels were comparable between gum addition treatments (results not shown) but, in all cases specific growth rates at 72 hours were higher at 150 rpm. At 150 rpm, these were 0.022, 0.017, 0.016 h^{-1} for gum concentrations 0, 0.5 and 1 g/L, respectively, while the corresponding values at 300 rpm were 0.012, 0.013 and 0.011 h^{-1}. The results suggest that the main observations discussed in the previous section regarding the shaker speed, remain valid independent of gum addition.

At 150 rpm, pellet formation tended to reduce with increasing gum concentration and no pellets appeared at 1 g/L. At 300 rpm, no gross morphological changes were detected by changing the viscosity and the mycelium was in all cases the free filamentous form. The effect of agitation was the same as with the shaker speed experiments of the previous section, however the medium viscosity appeared as yet another factor that influenced fungal morphology. Increased gum concentrations lower the dissolved oxygen levels in the medium. Increased dissolved oxygen concentrations have been

applied to induce mycelial pellet formation in fermentation cultures (*21)* and this obviously applies to the present case.

The effect of medium viscosity – Solid-State Fermentations

Solid-state fermentations from cultures of increased initial viscosity were successful when the precultures were grown at 150 rpm (Fig. 4). Although gum addition resulted in increased phytase activities in SmF at 300 rpm, the corresponding solid-state fermentations resulted in similar phytase activities from gum concentrations 0, 0.5 and 1 g/L, which were significantly lower compared to those obtained from inocula grown at 150 rpm.

Data analysis for the five sampling times of SSF (48, 72, 120, 144 and 168 h) revealed the following relationships: at 48 hours, the parameter shaker speed was significant (phytase levels reduced by 64 % going from 150 to 300 rpm) and there was speed/gum interaction. The 150 rpm cultures responded to gum concentration more than the 300 rpm cultures (decreasing phytase production at higher gum concentrations). At 72 h, shaker speed was significant and phytase levels reduced by 40 % going from 150 to 300 rpm. At 120 h, shaker speed was again significant and the reduction of phytase was 21 % going from 150 to 300 rpm. Phytase levels again reduced as gum concentration increased. At 144 h, shaker speed was significant; phytase levels reduced by 36 % going from 150 to 300 rpm, and phytase production was quadratic in response to gum concentration. Shaker speed/gum interaction was significant and again 150 rpm cultures responded to gum concentration more than the 300 rpm cultures. At 168 hours, shaker speed was significant, phytase levels reduced by 32 % going from 150 rpm to 300 rpm. Phytase production was quadratic in response to gum concentration. Shaker speed/ gum interaction was significant and 150 rpm responded to gum concentration more linearly than 300 rpm.

Estimation of the glucosamine content of the mycelium (Fig. 5) showed that growth levels in solid-state fermentations were markedly increased when inocula of 150 rpm cultures were used. Again, the increased specific growth rates of the 150 rpm precultures could be the reason for the increased growth and production on the solid substrate. It is known that in filamentous fungi, increased specific growth rates are associated with increased branching frequencies of the mycelium (*25,26*). This means that the total number of tips in the mycelium of increased growth rates would be higher com-

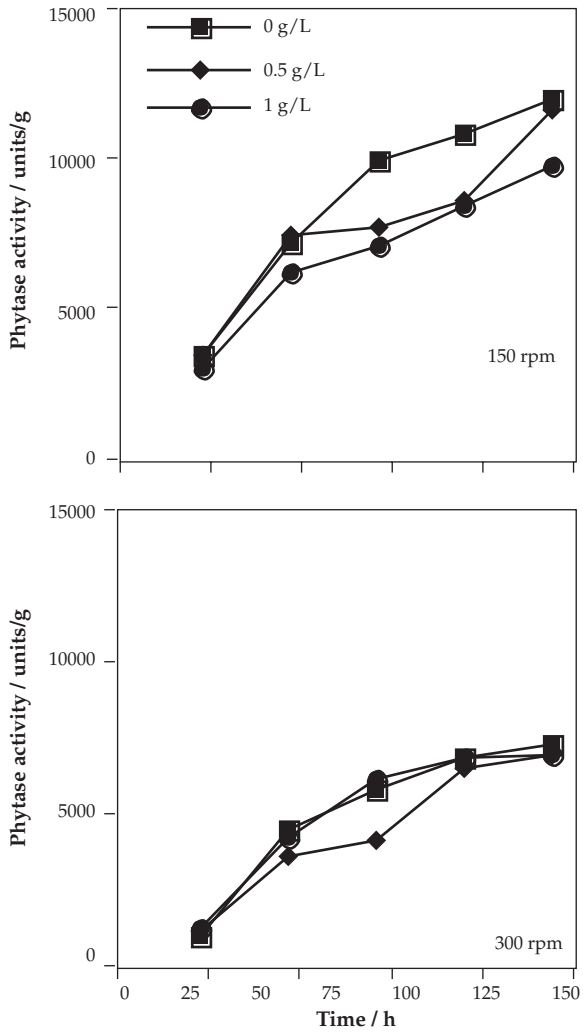

Fig. 4. Time courses of phytase activities in solid-state fermentation inoculated with cultures grown at 150 and 300 rpm and gum concentrations at 0, 0.5 and 1 g/L

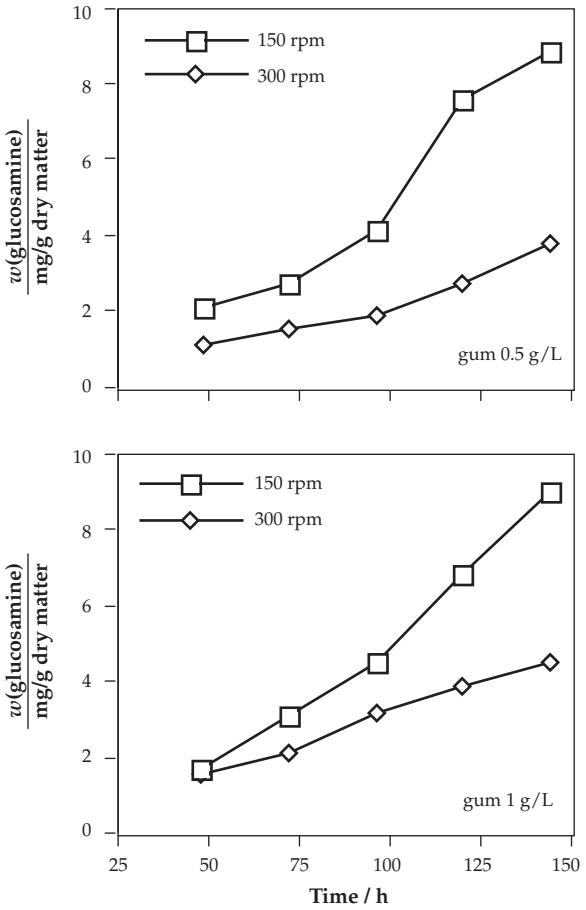

Fig. 5. Glucosamine content of the mycelium over time in solid-state fermentation inoculated with cultures grown at 150 and 300 rpm and gum concentrations 0.5 and 1 g/L

pared to that of lower specific growth rates. With regard to solid-state cultivation, this is an advantage since, under otherwise favorable conditions, an increased number of tips ensures that a larger proportion of the substrate will be attached to the mycelium.

Apart from the increased specific growth rates of precultures grown at 150 rpm, the phytase concentration at 72 hours was also higher in all gum concentrations in inoculum produced at this shaker speed. With the gum concentration 0.5 and 1 g/L, the phytase concentration at 150 rpm was almost 21 % higher than that of 300 rpm, at both gum conncentrations (Fig. 3). The corresponding phytase concentrations at 144 hours of solid--state fermentations were 34.1 % higher when the inoculum of 150 rpm and gum concentration of 0.5 g/L was used and 65.85 % higher when the inoculum of 150 rpm and 1 g/L gum was used (Fig. 4). However, when the inoculum phytase levels were plotted against SSF phytase production at 144 h, there is no evidence of correlation between the two. This is interesting because phytase production appears to be growth-associated (Figs. 1,2,4,5). Therefore the quality of inoculum is not determined exclusively by the quantity of biomass and further supports the importance of inoculum morphology for increased SSF productivities, while the phytase content of the inoculum may play a role under identical production conditions.

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Submerzna fermentacija i uzgoj na krutoj podlozi *Aspergillus niger* te proizvodnja fitaze

Utjecaj miješanja i viskoznosti medija na proizvodnju fitaze, na morfologiju plijesni i djelovanje inokuluma

Sažetak

Ispitan je kvalitativan odnos brzine miješanja i viskoznosti medija na morfologiju *A. niger* i proizvodnju fitaze pri submerznom uzgoju i uzgoju na krutoj podlozi. Povećavanjem brzine okretaja miješanja od 150 na 300 min^{-1} povećava se ukupna proizvodnja fitaze, iako su tijekom 72 sata nakon inokulacije specifične brzine rasta i proizvodnje fitaze veće pri 150 min^{-1}. Miješanje je znatno utjecalo na morfologiju plijesni tako da su pri 150 min^{-1} prevladavali mali peleti i mrežasti miceliji, dok su pri 300 min^{-1} dobiveni slobodni filamentozni oblici. Nakon inokulacije na krutoj podlozi postignuta je veća proizvodnja s inokulumom koji je rastao pri 150 min^{-1}. Pomak prema filamentoznom obliku rasta opažen je kada se u tekući medij dodala smola guar, čime se povećala viskoznost od 2 000 cp (podloga bez dodatka smole) na 52 000 cp (dodatak od 1 g/L smole). Pri objema brzinama miješanja, s jače izraženim utjecajem pri 300 min^{-1}, proizvodnja fitaze povećala se dodatkom smole. Specifična brzina rasta u prva 72 sata nakon inokulacije bila je veća pri 150 min^{-1}, bez obzira na koncentraciju smole, dok su fermentacije provedene na krutoj podlozi s tim kulturama dale veću količinu fitaze u usporedbi s onima dobivenim pri miješanju inokuluma sa 300 min^{-1}.