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Biomass estimation of *Aspergillus niger* $S_1$4 a mangrove fungal isolate and *A. oryzae* NCIM 1212 in solid-state fermentation

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Abstract

The suitability of protein and glucosamine measurements of cell components for biomass estimation of fungi *Aspergillus niger* $S_1$4, a mangrove isolate, and *Aspergillus oryzae* NCIM 1212 was evaluated using wheat bran as substrate in solid-state fermentation (SSF). Both direct as well as indirect methods were used. The protein and glucosamine contents and direct weight of the fungi were determined for 21 days and were found to vary significantly ($P<0.05$) with the duration of SSF. The direct measurement of biomass showed two distinct peaks on day 11 and 17 for *A. niger* and on day 12 and 17 for *A. oryzae*. In the case of the former, dry matter weight of the substrate gradually decreased as fermentation progressed with 68% loss on day 21. For the other species loss in dry matter content of substrate was only 30% on day 21. Glucosamine content showed three significant ($P<0.05$) peaks on day 3, 11 and 20 for *A. niger* and on day 3, 6 and 9 for *A. oryzae*. Protein content also showed three significant ($P<0.05$) peaks on day 2, 13 and 18 for *A. niger* and on day 2, 6 and 10 for *A. oryzae*. It has been concluded that the composition of the biomass as observed by measurement of protein and glucosamine contents typically varies during SSF and cannot be taken as standard procedures for biomass estimation. However, the results of the present study show that the process of SSF by *A. niger* for a period of 2-3 days can be applied for nutritional enrichment, four to six days for production of metabolites like enzymes and 6-21 days for bio-composting. *A. oryzae* can be effectively utilized for production of metabolites as well as nutritional enrichment.

Keywords: Solid state fermentation, *Aspergillus niger*, *A. oryzae*, biomass, protein, glucosamine

Introduction

SSF is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in the absence of free flowing liquid and has application in production of a wide variety of biomolecules, the most promising of which are enzymes, antibiotics, organic acids, aromatic compounds and enriched foodstuffs (Senez *et al.*, 1980; Barrios *et al.*, 1988; Tengerdy, 1996; Pandey *et al.*, 1999; Viccini *et al.*, 2001) The process of SSF upgrades the nutritional quality of agro industrial products and byproducts such as wheat straw, wheat bran, oil cakes and soybean meal that can be used in aqua feed and animal feed industries (Imelda-Joseph *et al.*, 2004). Filamentous fungi like *Aspergillus oryzae* plays a major role in the production of many commercial enzymes, such as á- amylase, glucoamylase, lipase and protease among others (Archer and Wood, 1995; Harvey and McNeil, 1994; Oxenbøll, 1994). It is also used to produce livestock probiotic feed supplements through fermentation (Kellems *et al.*, 1990; Gomez-Alarcon *et al.*, 1991). *A. niger* also has been extensively used in SSF for production of many enzymes (Tsdo *et al.*, 2000; Bhatnagar, 2004).

The quantity of biomass is an essential parameter in kinetic studies and for the characterization of the optimum growth and sporulation conditions for different fungi. In the case of solid-state fermentation, direct measurement of fungal biomass is hampered because fungi penetrate into and bind themselves tightly to the solid-substrate particles. Many authors have described indirect methods to estimate biomass in solid state fermentation based on measuring the content of certain cell components like chitin, ergosterol and protein (Arima and Uozumi, 1967; Matcham *et al.*, 1985; Desgranges *et al.*, 1991; Boyle and Kropp, 1992). The content of the different cell components can change markedly in fungi depending on fungal species, growth conditions and culture age (Oojikas *et al.*, 1998).

The objective of the present investigation was to determine changes in biomass of *Aspergillus niger* strain

$S_1$4 isolated from mangrove swamp and *Aspergillus oryzae* NCIM 1212 obtained from NCIM, Pune as measured by direct as well as indirect methods using wheat bran as the substrate in solid-state fermentation.

Materials and methods

The microorganisms used were *A. niger* strain $S_1$4 isolated from the soil samples of a local mangrove swamp at Cochin and *A. oryzae* NCIM 1212 an industrial strain obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India.

Wheat bran, procured from a local trader at Cochin, was used as the substrate for solid state fermentation. The bran was dried at 60 ±1°C for 24 h, ground and sieved (0.4 mm.) and used for fermentation.

The inoculum was produced by cultivation of *A. niger* and *A. oryzae* on potato dextrose agar (Hi-media, Mumbai) at 30±1°C and sub culturing was done once a fortnight. For the inoculum preparation, 10 ml of sterile Tween 80 (0.1%) was added to seven day old PDA slants and shaken well, and scraped with a sterile loop to make spore suspension (Smith *et al.*, 1996). The spore count in the suspension was estimated using a haemocytometer.

SSF was carried out in 500 ml conical flasks containing 20g wheat bran fortified with czapek dox to adjust the moisture content to 60% (autoclaved at 121°C at 15 lb for 20 minutes) (Czapek dox: $NaNO_3$ - 2.5 g l^{-1}; K_2HPO_4 - 1 g l^{-1}; $MgSO_4.7H_2O$ - 0.5 g l^{-1}; KCl - 0.5 g l^{-1}; *p*H @ 5.0) (Aikat and Bhattacharya, 2000). An inoculum size of 2 x 10^7 spores 20g wheat $bran^{-1}$ was used for each flask and incubated at 30 ± 1°C. All flasks were kept stationary at an initial *p*H of 6.4 - 6.5 for 21 days, with regular sampling at every 24 h interval starting from day 0 to day 21.

Direct biomass estimation : Duplicate samples of 1g fermented substrate from each flask were transferred to pre-weighed centrifuge tubes and 5 ml of sodium sulphate (150 g l^{-1}) was added to each tube. The tubes were centrifuged at 12000 rpm for 15 minutes. Centrifugation was repeated thrice under similar conditions to achieve complete separation of fungal mass from the substrate. At the end of centrifugation, the fungal mass with lower density than the substrate floated while the substrate settled to the bottom. The biomass alone was transferred to a pre weighed filter paper and dried in hot air oven for 72 h at 85±1°C to obtain a constant weight. Similarly, the substrate pellets in centrifuge tubes were also dried for 72 h and the weights were recorded. The remaining fermented substrate was dried at 85±1°C for 96 h to arrest further growth of the fungi and then stored at 50±1°C for determination of biomass using indirect methods.

Indirect biomass estimation: Glucosamine content was estimated following Ramachandran *et al.* (2005). Dried fermented sample (0.5 g) was taken in a 500 ml conical flask and 2 ml of concentrated sulphuric acid was added to it. This mixture was kept at 30°C for 24 h. It was then diluted with distilled water to make 1N solution and autoclaved at 15 lb pressure for 1h and filtered through Whatman no: 1 filter paper (Maidstone, UK) to remove the sediments. The solution was neutralized with 5 N NaOH and made up to 100 ml with distilled water. One ml of the sample was measured for absorbance at 530 nm against reagent blank (GENESYS UV 10 spectrophotometer). The standard used was n-Acetyl D-Glucosamine (Sigma, USA). The value was expressed as mg of glucosamine g^{-1} of dried fermented substrate (mg $gdfs^{-1}$).

Protein estimation was carried out following Lowry *et al.* (1951). The value was expressed as mg of protein g^{-1} of dried fermented substrate (mg $gdfs^{-1}$). The data obtained were analysed by two-way ANOVA. Pearson's correlation analysis was done to estimate the R-value and significance ($P<0.05$) of each variable determined.

Results

Biomass measurements: Measurement of biomass by direct method showed significant ($P<0.05$) variations during the 21 days of SSF by *A. niger* $S_1$4 and *A. oryzae* NCIM 1212 (Figs.1, 2). On day 11 and 17 two significant ($P<0.05$) peaks were obtained. Initially a gradual increase in biomass was observed till day 11 followed by a decline till day13 (0.34±0.01g) and again showed an increase to reach the maximum value on day 17 (0.57±0.01g) and thereafter showed a decline till day 21 (0.42±0.01 g). The growth was showing a logarithmic trend (R^2= 0. 809)

Biomass of *A. oryzae* also showed two significant ($P<0.05$) peaks during SSF, one on day 12 (0.24 ± 0.01g) and the other on day 17 (0.30± 0.006g), which was the maximum. The growth was showing a logarithmic trend (R^2= 0.7387)

Glucosamine: The glucosamine content of *A. niger* showed three significant ($P<0.05$) peaks on day 3 (11.83±0.15 mg $gdfs^{-1}$), day 11 (9.07±0.15 mg $gdfs^{-1}$) and day 20 (11.79±0.15 mg $gdfs^{-1}$) and that of *A. oryzae*

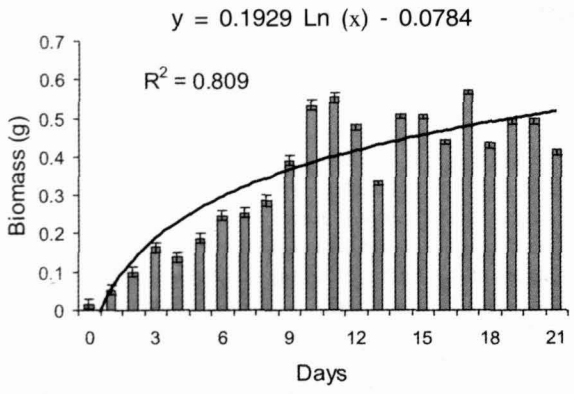

Fig. 1. Variation in direct biomass of A. niger *during 21 days of SSF*

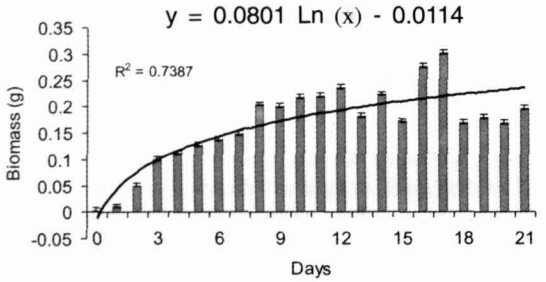

Fig. 2. Variation in direct biomass of A. oryzae *during 21 days of SSF*

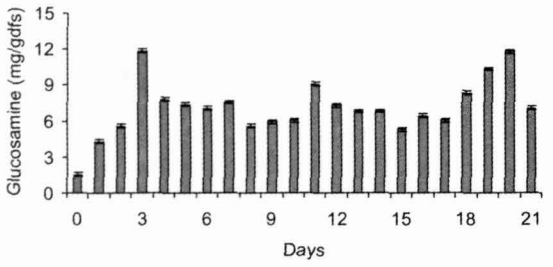

Fig. 3. Variation in glucosamine content of A. niger *during 21 days of SSF*

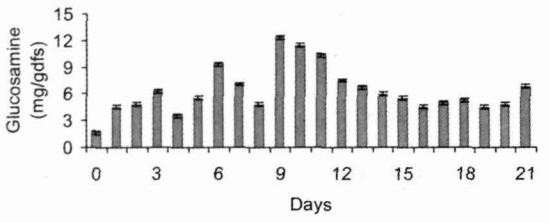

Fig.4. Variation in glucosamine content of A. oryzae *during 21 days of SSF*

showed a gradual increase upto day 3 (Figs.3, 4), but a declining trend was observed on day 4 (3.53±0.2 mg $gdfs^{-1}$) followed by an increase with a significant ($P<0.05$) peak on day 6 (9.32±0.2 mg $gdfs^{-1}$). On day 9, glucosamine content reached the maximum of 12.34±0.2 mg $gdfs^{-1}$.

Protein: Protein content of both the strains showed significant ($P<0.05$) variation with duration of fermentation. Protein content was the maximum (300±3.08 mg $gdfs^{-1}$) on day 2 for *A. niger* (Fig.5) and on day 10 for *A. oryzae* (Fig. 6) (255.8±2.5 mg $gdfs^{-1}$), which afterwards showed a declining trend till day 20 (55.8±2.5 mg $gdfs^{-1}$). On day 21 it further increased to 180.4±2.5 mg $gdfs^{-1}$.

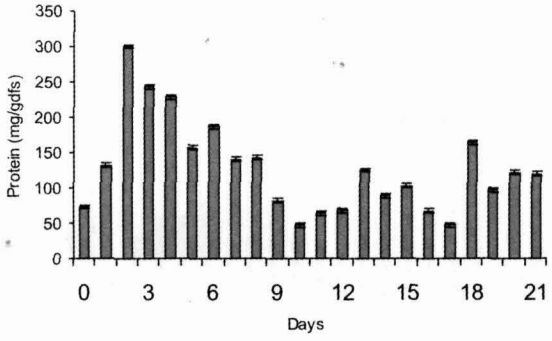

Fig. 5. Variation in protein content of A. niger *during 21 days of SSF*

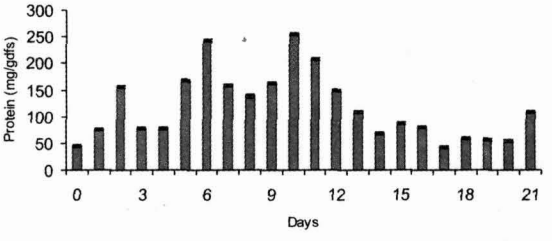

Fig. 6. Variation in protein content of A. oryzae *during 21 days of SSF*

Substrate utilization and biomass: In the case of *A. niger* the substrate utilization was gradual with the progress of fermentation, and was significant ($P<0.05$) with duration (Fig. 7). From day 10 the quantity of substrate became lesser than the biomass. Whereas, on day 13 it was observed that the biomass (0.34± 0.01g) was less than the substrate (0.44± 0.01g). From day 11 onwards, substrate utilization was higher compared to biomass production. About 68% reduction in substrate was observed from day 0 to day 21.

In the case of *A. oryzae* the substrate utilization was significant (P<0.05) with the fermentation duration, but the substrate content was always higher than biomass (Fig. 8). About 30% reduction in substrate was recorded after 21days of fermentation.

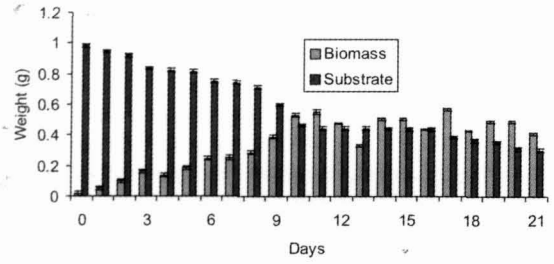

Fig.7. Variation in substrate and biomass of A. niger *during 21 days of SSF*

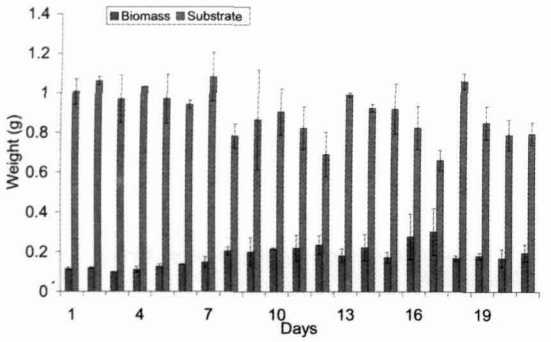

Fig. 8. Variation in substrate and biomass of A. oryzae *during 21 days of SSF*

Discussion

During solid-state fermentation, many processes like, substrate consumption, biomass production and evaporation of water take place simultaneously. Wheat bran fortified with czapek dox provided adequate nutrients for the fungi during SSF at constant initial temperature (30±1°C), *p*H (6.4±0.1) and moisture (60%). The direct biomass measurement of the two selected fungal species resulted in typical sigmoid pattern (Figs. 1, 2). It was observed in the present study that the dry matter weight of the substrate gradually decreased as the fermentation progressed in the case of *A. niger*, which indicated the gradual utilization of substrate for biomass and energy production. The weight of substrate became lesser than the fungal biomass from day 10 onwards (Fig. 7). *A. oryzae* followed a different pattern of growth wherein the dry matter content of substrate always exceeded the fungal biomass (Fig. 8). This may be due to the difference in production of metabolites during the growth phases of the two species. *A. niger* degraded substrate at a faster pace than *A. oryzae* which resulted in almost two fold reduction of the substrate. This may be the reason why *A. niger* finds application in composting (Anjaneyulu and Bindu, 2000; Pau and Omar, 2004). During filamentous fungal growth on solid substrates, it is generally assumed that there is a limitation in the oxygen supply to the cells that are in close contact with the substrate, which is elementary for substrate utilization (Oostra *et al.*, 2001). Oxygen is mainly (70%) taken up by the aerial hyphae of *A. oryzae* (Rahardjo *et al.*, 2001). Moreover, there is a lack of oxygen supply to the penetrative hyphae during growth on a solid substrate. The variation in substrate utilization in the present study can be attributed to the difference in efficiency of the strains and the variation in cell components and metabolites during different phases of fungal growth.

In the case of SSF, direct estimation of fungal biomass is hampered because fungal hyphae penetrate into and bind themselves tightly to the solid substrate particles (Ooijkaas *et al.*, 1998, 2000), which often makes it necessary to use indirect methods of determining fungal biomass in SSF, such as measurement of cell components (Raimbault, 1998; Viccini *et al.*, 2001). The present observations on cell components included glucosamine and protein contents of fungal cells.

The composition of the biomass typically varies during fermentation as observed for protein and glucosamine contents (Figs. 3, 6). Since the evolution of biomass dry weight in time cannot be accurately measured in SSF, determination of glucosamine content was reported as the most promising one (Smits, *et al.*, 1998). Glucosamine content of fungi in the present study showed a varying pattern during SSF (Figs. 3, 4). Glucosamine content showed three significant (P<0.05) peaks on day 3, 11 and 20 for *A. niger* and on day 3, 6 and 9 for *A. oryzae* The concentration of glucosamine in biomass, however, might vary with time and culture conditions (Kim *et al.*, 1985; Desgranges *et al.*, 1993).

Protein contents also showed three significant (P<0.05) peaks on day 2, 13 and 18 for *A. niger* and on day 2, 6 and 10 for *A. oryzae* (Figs. 5, 6). The increase in protein content during initial days of fermentation itself suggests the growth of fungi contributing to the fungal protein by utilizing available nutrients in the substrate. The fungus was able to metabolize the available protein in the substrate, and therefore the shape of the protein profile against time is the result of two opposing pro-

Table 1. *Two-way ANOVA for biomass over days and species*

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
SPECIES	1.06729	1	1.06729	8143.16826	0.00000
DAYS	1.96489	21	0.09357	713.88844	0.00000
SPECIES*DAYS	0.43403	21	0.02067	157.69136	0.00000
Error	0.01153	88	0.00013		

Durbin-Watson D Statistic 2.863
First Order Autocorrelation -0.446

Table 2. *Two-way ANOVA for protein over days and species*

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
SPECIES	2942.12180	1	2942.12180	1.23063E+07	0.00000
DAYS	2.69847E+05	21	12849.84789	5.37484E+07	0.00000
SPECIES*DAYS	2.57990E+05	21	12285.25309	5.13869E+07	0.00000
Error	0.02104	88	0.00024		

Durbin-Watson D Statistic 2.889
First Order Autocorrelation -0.447

Table 3. *Two-way ANOVA for glucosamine over days and species*

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
SPECIES	20.79587	1	20.79587	82850.21556	0.00000
DAYS	434.91405	21	20.71019	82508.86076	0.00000
SPECIES*DAYS	320.34852	21	15.25469	60774.28772	0.00000
Error	0.02209	88	0.00025		

Durbin-Watson D Statistic 2.899
First Order Autocorrelation -0.457

Table 4. *Two-way ANOVA for substrate over days and species*

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
SPECIES	1.37588	1	1.37588	10855.91830	0.00000
DAYS	3.51057	21	0.16717	1318.99900	0.00000
SPECIES*DAYS	0.48786	21	0.02323	183.29838	0.00000
Error	0.01115	88	0.00013		

Durbin-Watson D Statistic 2.849
First Order Autocorrelation -0.429

cesses: the consumption of substrate protein and the production of biomass protein (Viccini, *et al.*, 2001). The reduction in protein content in the later phases of fermentation suggests that probably when the readily available nutrients get depleted the degradation of complex molecules of the substrate by the fungi follows. Raimbault and Alazard (1980) also observed that maximum enzyme production and early declining phase were achieved much faster due to the rapid degradation of substrate as a consequence of rapid growth. Nevertheless, the degradation products, which were gradually forming, were used up by the fungal biomass to enhance protein production later in the fermentation process. The studies by Carlsen *et al.* (1996) suggested that enzyme production was closely connected to the growth of the fungus, while enzyme synthesis was tightly regulated by the concentration of glucose in the cultivation medium. The three different peaks observed in protein content may be attributed to the change of biomass from fungal hyphae to more of fungal spores, for which precise determination of protein content is difficult due to the thick spore coat. The composition of the biomass typically varies during fermentation and this has been noted for protein and glucosamine contents (Mitchell *et al.*, 1991; Nagel *et al.*, 2001). From the results, it was observed that, protein and glucosamine

contents of *A. niger* and *A. oryzae* followed similar patterns, whereas the pattern was different for direct biomass. At present the only method available to take this into account is to determine the variations as a function of time in an artificial system that allows biomass determination, and to presume that the same variation occurs in the real system. Such an approach was taken by Nagel *et al.* (2001) who arrived at an equation for glucosamine content of the biomass (G_x) mg glucosamine mg dry biomass^{-1} as a function of time.

Although the first industrial use of partially purified enzyme preparations of filamentous fungi involved *A. oryzae* in a solid state fermentation process (Takamine, 1914), fundamental knowledge about the microbial biology and growth kinetics of this process is limited compared to the growth of filamentous fungi in submerged fermentation. However, recently, increasing research attention is given to studies on growth of filamentous fungi on solid substrate because of its high protein production capacity (Te-Biesebeke *et al.*, 2002; Holker *et al.*, 2004).

From the results of the present study it can be observed that by using *A. niger* in SSF, the initial phase (2-4 days) can be utilized for nutritional enrichment of substrate when fungal protein content is high and substrate contents is less used up, then (4-6 days) for production of metabolites when less variation in biomass is recorded and later phase (6-21 days) for bio-composting when the substrate volume gets reduced. As per earlier studies it has been reported that in SSF nutritional enrichment of substrate is attained in the first phase (Vijayakumar, 2003) and enzyme production is accomplished in the second phase (Bhatnagar, 2004). At the final stage when the substrate gets considerably used up and reduced into simpler molecules *A. niger* has been recommended for use in bio-composting (Anjaneyulu and Bindu, 2000; Pau and Omar, 2004).

Biomass is a fundamental parameter in the characterization of microbial growth. Its measurement is essential for kinetic studies on SSF (Raimbault, 1998). The metabolic activities such as substrate consumption and product formation are strongly related to either or both of the growth rate and the actual biomass present. Understanding the variations in biomass components and their interactions can enable designing new models for SSF bioreactors.

In order to make growth models particularly beneficial in solid state fermentation processes, further research is needed in various domains like converting indirect measurements into reliable estimates of biomass and understanding the interaction of varying environmental conditions of SSF on the growth kinetics of the fungi.

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