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Research Article

A Novel Spectrometric Method for Fungal Growth Estimation

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Abstract

Enzyme producing industries majorly use fungus based submerged fermentation systems. The paper discusses an innovative technique based on Optical Density measurement at 450 nm to estimate the growth of *Trichoderma harzanium* ATCC 20846. A step of sonication and homogenization was performed before the OD at 450 nm estimation in a spectrophotometer. *Trichoderma harzanium* ATCC 20846 was grown in a defined cellulose containing media and in Potato Dextrose Broth. The OD at 450 nm and the dry mycelia weight were simultaneously measured during the growth. For the growth in Potato Dextrose Broth, the relationship is 1 OD at 450 nm = 4.24 ± 0.23 g dry mycelial weight. For the fungal growth in a defined Vogel's media having the insoluble substrate cellulose, the relationship is 1 OD at 450 nm = 3.94 ± 0.15 g dry mycelial weight. This method is time saving and has economic viability when compared to the existing complicated and time consuming methods of fungal growth estimation.

Keywords: Mycelia; Cellulose; Optical Density; Submerged fermentation; Spectrophotometer.

Introduction

Biotechnology is rapidly gaining interest due to its capability to produce value based products using wild type and genetically modified organisms. Fermentation or Submerged fermentation as it is technically called is the preferred method for the continuous production of the value added products. Cellulases and xylanases are gaining interest as they are broadly used in the food and feed, the textile industry, and the pulp and paper industries [1]. The capability of the filamentous fungi to secrete consortia of proteins has branded them the industrial workhorse for the production of several industrially important enzymes. *T. harzanium* is an asexually reproducing fungus commercially used as a fungicide. It belongs to the Division Ascomycota and the family hypocreaceae. Asexual spores are majorly seen while sexual reproduction has also been reported in *T. harzanium.* The fungus is also widely used in the biofuels area as it is a producer of cellulases. In submerged fermentations, the morphology of the filamentous fungi varies between pellets and dispersed mycelia, based on their growth conditions and the genotype of the microbial strain [1]. In fungal cultures, when the growth commences inside the fermentor, the mycelia abruptly grows during the initial stages and maintains biomass towards the end of the fermentation [2].

For filamentous fungi, in general, five clear growth phases are observed. The initial lag phase, the first transition phase, the log phase, the second transition phase, and the stationary phase are the five phases [3]. In the stationary phase, when all the nutrients are depleted, the fungus starts producing enzymes [4]. When cultivated in a nutrient rich media, each strain exhibits a small variation in the log phase [3]. The filamentous and the non-homogenous filamentous growth of fungi make the analysis of growth characteristics by growth curves an ordeal [3]. A possibility for the measurement of the growth of fungi using the Optical Density (OD) measurement has been proposed by [14]. A brief introduction on the currently followed techniques of growth analysis of a filamentous fungus and the reasons for skepticism in their usage are listed below. The membrane lipid ergosterol is unique to fungi. For biomass estimation mycologists study the content of ergosterol, which is considered to be rapidly degrading after the death of the fungi. For the ergosterol based estimation, fungus samples

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from various environments were collected and assessed for ergosterol content. But it has been observed that the ergosterol did not degrade rapidly in contrary to the general assumption. Instead it resided for a longer time in the fungal biomass, even after death of the biomass. After a complex process of drying the biomass, lyophilizing them and extracting the ergosterol by refluxing, the content of ergosterol needs to be evaluated using a HPLC. This technique, though initially adapted by many researchers, eventually faded away due to the time consuming and complex extraction procedures. The general assumption of ergosterol being labile has also been disproved [5].

The fungus is grown separately in Erlenmeyer flasks. During the growth, a calculated quantity of the culture broth along with the mycelia is recovered using a Buchner funnel and dried at 70 degree Celsius for 24 hours. A plot of the Dry cell weight vs the time of incubation is made to construct a growth curve [6]. The method is suitable for the dry cell weight determination. But the method may not be suitable while insoluble substrates such as micro-crystalline cellulose is being used. The media component may stick to the filter paper showing false weight figures during the dry cell weight analysis. For the Mycelial Dry Weight Determination method, the dry cell weight and the total protein concentration in the fermentation broth is determined. The protein content is estimated using the Lowry's method. A relation is established between the protein content and the dry cell weight [6]. Some scientists still use this method of growth estimation.

Growth Estimation Based on the Average Hyphal Length is performed using microscopy. The average length of the hyphae with respect to time is calculated [3]. Once the new conidia are formed and they germinate, keeping track of the exisiting hyphal length becomes implausible. Resolution issues with microscopes worsen the estimation, furthermore. Swabs are collected from 7-10 day old slants. After counting the spores on a haemocytometer, the spores are inoculated into microtitre plates of 100 microliter culture volume. The OD is studied constantly at 405 nm throughout the period of growth to estimate the increase in the biomass concentration [3]. OD measurements of growth in microtiter plates are also used to quantify the increase in the population. Baranyi and Roberts predictive mathematical model has been used to standardize this method [7]. In a slightly modified method, the growing fungi in microtiter plates are proportionally incubated into cuvettes and the growth is constantly monitored using a double-beam spectrophotometer [8]. In another method to measure the spore concentration of the fungus, the spore count in the haemocytometer is related to the OD measurement [9]. C14 labeled glucose is introduced in a pre-determined concentration into the fungus culture's nutrient media. Samples are aliquoted and checked for the change in radioactivity using a radio spectrometer. The C14 incorporation is related to changes in the accumulated mycelium. However, this Radioactively Labelled Substrates method showed certain inconsistencies [8].

Materials and Methods

Materials

Spectrophotometer (M/s Shimadzu UV 2600 Model), Homogenizer (M/s GEA Niro Panda Plus 2000 model), Sonicator (M/s Vibra cell 750 Watt Model) were the equipments used for the experiments.

The strains were purchased from The ATCC. The nutrient rich media Potato Dextrose Broth procured from M/s Hi-Media was used for the growth of fungi. It was prepared according to the manufacturer's instructions and autoclaved at 121°C for 20 min. Other reagents used below were procured from the brand Hi-Media.

Submerged fermentation of Trichoderma harzanium ATCC 20846

The composition of Vogel's media (g/L) is as follows: Tryptone (1 g/L), Tri-sodium citrate (2.5 g/L), Di-Potassium hydrogen phosphate (5 g/L), Ammonium nitrate (2 g/L), Magnesium sulphate heptahydrate (1.4 g/L), Microcrystalline cellulose 1% (w/v), Calcium chloride dehydrate 0.1 g/L, Tween 80 – 0.2 % (v/v). A Trace element solution containing Citric acid monohydrate 5 g/L, Zinc sulphate heptahydrate 5 g/L; ferrous ammonium sulphate 1 g/L, Copper sulphate 250 mg/L, Manganese sulphate 50 mg/L ; Boric acid 50 mg/L, Sodium molybdate 50 mg/L was prepared. 1 mL of trace elements per litre of the total media components was added [11]. The pH was set at 5.5 before autoclaving.

A calculated spore suspension of ($2x10^9$ spores/mL) was inoculated into two primary culture flasks. 100 mL of Potato Dextrose Broth was inoculated with the *T. harzanium* spores and it was cultured for 4 days. In another flask containing 200 mL of 1% Microcrystalline cellulose with Vogel's media, spores were inoculated and cultured for 5 days. A 3 litre Hopkins flask containing 700 mL of Potato Dextrose Broth was inoculated with primary culture from the PDB flask (10% v/v inoculum). A 5 litre flask containing 1 litre Vogel's media with 1% Microcrystalline cellulose was inoculated with the primary culture in 1% Microcrystalline cellulose containing Vogel's media (15%, v/v inoculum). The flasks were incubated in a shaking incubator at a temperature of 28 degree Celsius with 180-200 rpm shaking. The method of growth estimation based on the OD at 450 nm is a slightly modified version of the method developed by Banerjee et al [14]. The experiment was performed to establish a relationship between the conventional method of estimating the dry mycelial weight of the fungus during various phases of growth and the proportional increase in the Optical Density values at 450 nm. It was performed in a nutrient rich media such as Potato Dextrose Broth and an enzyme production media such as Vogel's with 1% Microcrystalline cellulose to estimate the difference in growth profiles and biomass formation of *T. harzanium* ATCC 20846 in both the media.

OD_{450} Estimation for the Fungal Culture

Around 30 mL samples were collected each time from both the flasks. The samples were sonicated at 40% amplitude over an ice bath for a cycle time of 20 min with a 30 sec pulse and a 5 second gap in between each pulse. Temperature of the sonicator probe was not allowed to exceed 40°C (monitored using a temperature probe provided with the setup). Following this, the samples were homogenized at 600 bar using a Homogenizer [15]. The samples were introduced into the equipment at room temperature. Homogenized samples were kept in sterile falcon tubes placed in an ice bath. The OD of the homogenized sample was measured at 450 nm. For the fungus grown in the Potato Dextrose Broth, a sterile sample of the same was used as the spectrophotometer blank while a sterile sample of 1% Microcrystalline cellulose containing Vogel's media was used as the spectrophotometer blank for the fungus grown in the cellulose containing media.

Mycelial Dry Weight Measurement

The dry weight of the mycelia from the culture contained in the Potato Dextrose Broth media was estimated using a Whatmann No. 1 filter paper by filtering 50 mL of the culture broth and drying the contents accumulated in the filter paper for 24 hrs at 40°C in a hot air oven [4,8,16]. To measure the dry mycelial weight of the fungus growing in the media containing the insoluble Microcrystalline cellulose substrate, a slightly modified version of the Updegraff's method was followed [1,12].

Mycelial Dry Weight Measurement of the fungus grown in PDB Media

50 mL of the culture broth was collected and centrifuged for 20 minutes at 9000 rpm. The supernatant was discarded. The pellets were washed with 10 mL Distilled water for 3 times. Following this the pellets were collected on a pre-weighed Whatmann No. 1 filter paper [12] and dried for 24 hours at 40 degree Celsius in a hot air oven. This is a slightly modified protocol used by Willian et al [1].

Mycelial Dry weight = (Mycelial Dry weight – empty filter paper weight) g

Dry Weight – Dry weight of the mycelia after 24 hour drying at 40°C in g.

Empty filter paper weight – Weight of the empty Whatmann filter paper measured before filtration in g.

Mycelial Dry Weight Measurement for fungus grown in Vogel's media with insoluble cellulose

Two sets of samples designated as sample A and sample B were collected during the same time points to evaluate separately the mycelial mass and the amount of unused cellulose substrate.

Sample A: To estimate the total fungal biomass, 50 mL of the culture broth was collected and it was centrifuged for 20 min at 9000 rpm. The supernatant was discarded. The pellets were washed with 10 mL Distilled water for 3 times. Following this the pellets were collected on a pre-weighed Whatmann No. 1 filter paper and dried for 24 hours at 40 degree Celsius. This dry weight is the weight of the mycelia inclusive of the unconsumed insoluble cellulose substrate.

Sample B: To estimate the unconsumed insoluble cellulose, 50 mL of the total culture was centrifuged at 5000 rpm for 20 min and the supernatant was aspirated out. The pellets were suspended in acetic acid–nitric acid reagent (15 mL:150 mL of 80% acetic acid with 15 mL of pure nitric acid) and boiled for 30 min in a water bath. After cooling and centrifuging at 5000 rpm for 20 min, the pellets were washed with distilled water 10 mL, and the residual cellulose was dried at 40 degree Celsius for 24 hrs [13].

Sample A: Dry Weight Measurement

Sample A Dry weight = [Dry weight – empty filter paper weight] g.
Sample A along with the mycelial mass' weight also includes the weight of the insoluble cellulose substrate.
Dry Weight – Dry weight of the centrifuged Sample A after 24 hrs drying at 40°C in g.
Empty filter paper weight – Weight of the empty Whatmann filter paper measured before filtration in g.

Sample B: Dry Weight Measurement

Sample B Dry weight = [Dry weight – empty filter paper weight] g.
Sample B after acid treatment would have lost all the mycelia and retains only the insoluble cellulose substrate. Sample B gives the weight of the unconsumed cellulose.
Dry Weight – Dry weight of the centrifuged acid treated Sample B after 24 hrs drying at 40°C in g.
Empty filter paper weight – Weight of the empty Whatmann filter paper measured before filtration in g.
Dry weight of the mycelia = (Dry weight of Sample A – Dry weight of Sample B) g

Results and Discussion

Growth Estimation for Fungus Grown in Potato Dextrose Broth

In PDB, the fungus multiplied very fast. At the end of 12 hrs, filamentous hyphae were observed and the media turned slightly turbid. The samples collected were sonicated, homogenized and the OD was read at 450 nm. The growth inside the flask was observed to be quick. The mycelia formed as non-homogenous and filamentous structures. Homogenization and sonication steps help in increasing homogeneity of the culture broth. The turbidity increased steeply until 48 hours. A slight constancy in the OD values was obtained after 72 hrs. After 96 hrs until 168 hrs the OD values increased in a gradual trend. Constancy in the growth was observed for two time points of growth after 168 hours as shown in fig. 1. A maximum OD of 1.325 ± 0.44 was obtained after 168 hours indicating a high biomass increase. This increase in OD correlated to a very high density of the hyphal growth. After 168 hours the OD values did not increase significantly. Potato Dextrose Broth is an optimized media for fungal growth. Biomass buildup is the objective of growth in PDB. In our case, to estimate the variation in biomass accumulation in a growth media and a production media we have used PDB and a enzyme production media containing cellulose. Maximum growth has been observed in PDB containing flasks. At the end of the growth, the whole media turned turbid, indicating a very high increase in the biomass. The estimation of dry mycelia weight confirmed the growth increase.

50 mL sample was withdrawn from the conical flask which contained the culture. The biomass increase was high from 24 hrs to 48 hrs. From 72 hrs to 168 hrs, the biomass increased in size and accumulated as a dense mass inside the flask. A small constancy observed between 48 and 72 hrs may be due to the resistance to mass transfer. The media became turbid and dense once the mycelia started accumulating. The biomass settled down to the bottom of the flask. The oxygen from the top liquid layer would take time to diffuse to the bottom of the flask. Though a high rate of agitation of 180-200 rpm (optimized) was maintained, the sudden burst in the biomass increase and a depletion of the oxygen in the headspace may have caused constancy in growth. A higher rate of agitation than that followed here causes disruption of the biomass and it ruptures. A lower agitation causes improper aeration and no growth is observed until 72 hrs. A bottom aerated vessel may not have caused this issue. In a flask based growth, unlike an aerated reactor, the oxygen in air passes to the flask only when the oxygen in the headspace is consumed and a fresh volume of air from the atmosphere moves in through the cotton plug. After 72 hrs, a gradual increase was observed in the biomass concentration as shown in fig. 2. The biomass concentration increased gradually to 5.2 ± 0.33 g/L at the end of 168 hrs. For growth in PDB, the relationship proposed is

1 OD at 450 nm = 4.24 ± 0.23 g dry mycelial weight as shown in table 1.

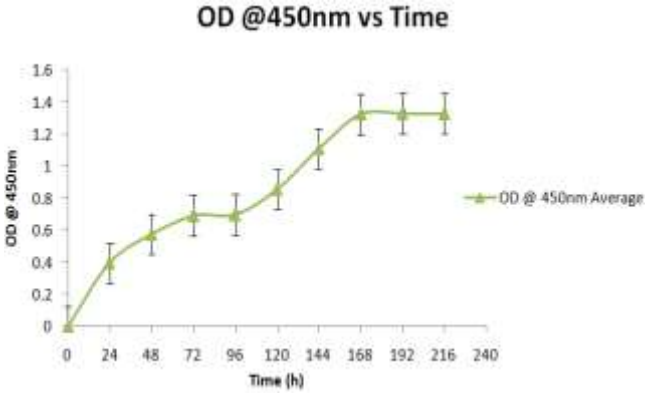

Fig. 1. Optical Density at 450nm vs Time (hours) for growth of *T. harzanium* in Potato Dextrose Broth

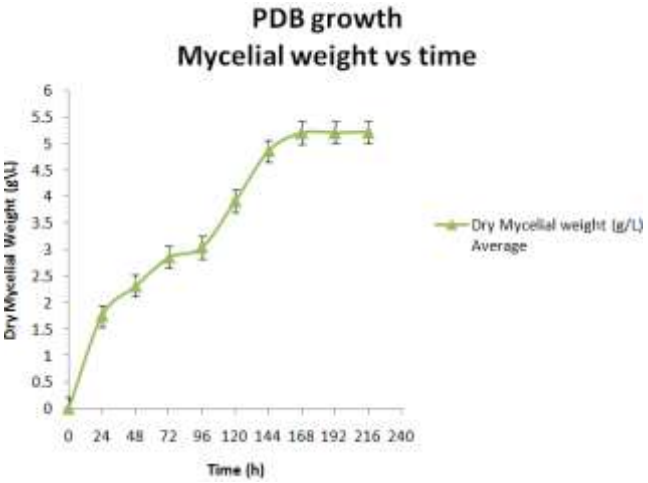

Fig. 2. Dry Mycelial weight vs Time for growth of *T.harzanium* in Potato Dextrose Broth

Table 1. A tabulation showing the time of growth of *T. harzanium* in PDB and the OD measured at 450 nm and the dry mycelia weight

S. No.	Time (hrs)	Dry Mycelial weight (g/L)	OD at 450 nm
1	24	1.75 ± 0.29	0.398 ± 0.13
2	48	2.32 ± 0.05	0.576 ± 0.08
3	72	2.86 ± 0.14	0.692 ± 0.125
4	96	3.04 ± 0.25	0.699 ± 0.035
5	120	3.92 ± 1.24	0.856 ± 0.048
6	144	4.86 ± 1.20	1.111 ± 0.021
7	168	5.20 ± 0.33	1.325 ± 0.449

Fungus Growth in Vogel's Media having an Insoluble Substrate

The Filamentous fungus culture was collected and the OD was estimated at 450 nm. After a step of sonication and homogenization, the OD was estimated. Sterile media solution was used as the blank. Until 24 hrs, the accumulation of biomass was very slow. After 24 hrs, the hyphal concentration steeply increased until 48 hrs. After 48 hrs there was a mild increase in viscosity of the media due to the production of cellulases by the fungi. The viscosity may hinder mass transfer and this may be attributed to the very mild increase in biomass from 48 to 72 hrs as shown in fig. 3. The broth showed non-netwonian characteristics. An increase in the rate of agitation causes increase in viscosity which conveys that the fluid possesses dilatant fluid characteristics. This increase in viscosity happens because the accumulated hyphae are disrupted by the shear caused due to agitation. The cellulases accumulated inside the hyphal tip spread out after the hyphal rupture. Increase in agitation disturbs mature mycelia. Newer mycelia would further require another phase of adaptation before they mature and start consuming the cellulose. Hence an optimal agitation of 180-200 rpm is maintained throughout the process. Since, the cellulose media is an enzyme production media, the focus of the growth is the production of cellulases rather than the accumulation of more biomass. This is the reason for the difference in the fungus growth characteristics between the two media. The maximum OD value at 450 nm obtained at the end of 168 hours is 0.969 ± 0.09 which is slightly lesser than that obtained for the growth in PDB. After 168 hours, there was no significant increase in the OD_{450} values.

Mycelial Dry Weight Analysis

The mycelial dry weight was analyzed according to a slightly modified version of the method postulated by [12]. Initially, the total weight measured included the weight of the cellulose as well. So in a similar quantity of the sample, a step of acid addition eliminated the mycelial biomass. The dry weight of the sample obtained after acid addition is the weight of the unconsumed insoluble cellulose. The total weight of the sample including the cellulose and mycelia is subtracted from the cellulose weight to obtain the weight of the mycelia alone. In a defined media or production media as we usually term it, the fungus takes a while to adapt to the new carbon source. It had a lag phase were slow growth was observed until 24 hrs. This phase of adaptation will stimulate the enzyme machinery inside the host fungi. In order to solubilize and

metabolize the carbon source, the fungus will secrete certain enzymes (cellulase in this case).

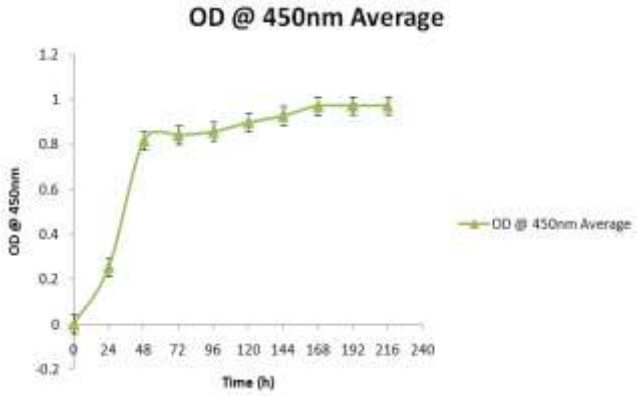

Fig. 3. Optical Density at 450 nm vs Time (hours) for growth of *T. harzanium* in 1% Microcrystalline cellulose in Vogel's media

The environmental stress levels increase as the available carbon source is tough to metabolize. Under such stress conditions, the cAMP levels within the cells will rise and following a sequence of events high concentrations of enzymes are produced by the host to metabolize the insoluble substrate. Cellulose uptake was steep after 24 hrs until 48 hrs. After 48 hrs the uptake of cellulose was progressively incremental and not as abrupt as observed between 24th hr and 48th hr (Fig. 4). The biomass concentration observed was 3.68 ± 0.36 g/L at the end of 168 hrs. After 168 hours, the mycelia weight did not increase much as shown in fig. 5. A portion of cellulose was left behind unconsumed. The presence of an easily metabolizable carbon source such as glucose or glycerol will cause a phenomenon called catabolite repression where the enzyme production is hampered greatly [10]. It is at this stage that a residual concentration of 2 ± 0.05 g/L cellulose was left behind unused as shown in fig. 6. The fungus utilizes the produced glucose instead of cellulose. However, the biomass concentration and the OD values did not increase much after 168 hrs. The presence of glucose in the broth was estimated using the DNSA reducing sugar method. While a sterile sample of Vogel's media containing 1% Microcrystalline cellulose did not show the presence of reducing sugars such as glucose, the samples from the culture after 168 hrs showed the accumulation of reducing sugar glucose. The cellulases produced by the fungus had hydrolyzed the celluloses to their monosaccharide component glucose. Hence, a condition of non-availability of reducing sugars should be maintained throughout in submerged fermentations to promote complete cellulose utilization and enzyme production [14]. For the fungal growth in a defined media having the insoluble substrate cellulose, the relationship is 1 OD at 450 nm = 3.94 ± 0.15 g dry mycelial weight as shown in table 2.

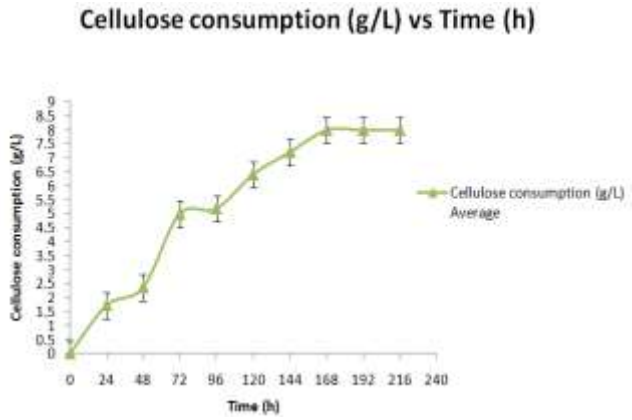

Fig. 4. Cellulose Consumption Vs Time for growth of *T. harzanium* in 1% Microcrystalline cellulose in Vogel's media

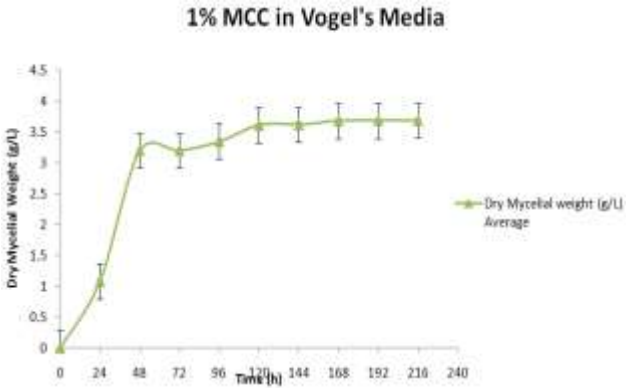

Fig. 5. Dry Mycelial weight Vs Time for growth of *T. harzanium* in 1% Microcrystalline cellulose in Vogel's media

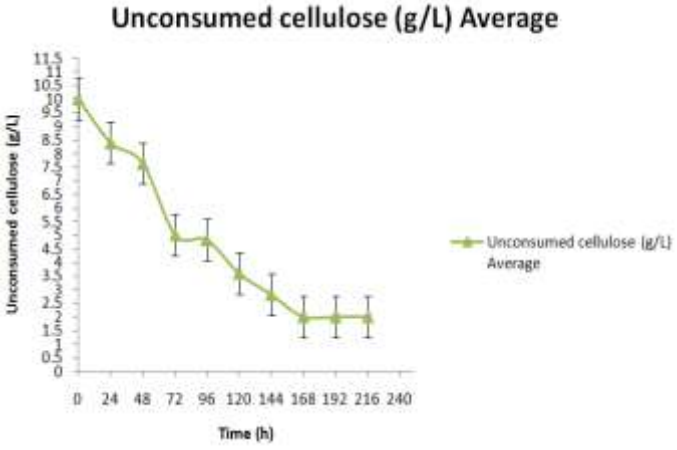

Fig. 6. Cellulose utilization vs Time for growth of *T. harzanium* in 1% Microcrystalline cellulose in Vogel's media

Table 2. A tabulation showing the time of growth of *T. harzanium* in 1% cellulose containing Vogel's media and the OD measured at 450 nm and the dry mycelia weight

S. No.	Time (hrs)	Dry Mycelial weight (g/L)	OD at 450 nm
1	24	1.08 ± 0.12	0.254 ± 0.04
2	48	3.20 ± 0.29	0.816 ± 0.04
3	72	3.20 ± 0.09	0.840 ± 0.02
4	96	3.35 ± 0.42	0.856 ± 0.03
5	120	3.61 ± 0.18	0.896 ± 0.05
6	144	3.62 ± 0.52	0.926 ± 0.03
7	168	3.68 ± 0.36	0.969 ± 0.09

Conclusion

The work was performed with the main objective of establishing a relationship between the conventional mycelial dry weight estimation and a novel method of measuring the Optical Density of the fungus at 450 nm after homogenizing the culture samples. Since the growth characteristics of a fungus varies in between a growth enhancing nutrient rich media and an enzyme production media containing an insoluble substrate cellulose along with certain minimal essential media components, we have performed analyses of the growth patterns of *Trichoderma harzanium* ATCC 20846 on both the media. Two different relationships for the two media components have been proposed in the work. For the growth in Potato Dextrose Broth, the relationship is 1 OD at 450 nm = 4.24 ± 0.23 g dry mycelial weight. For the fungal growth in a defined Vogel's media having the insoluble substrate cellulose, the relationship is 1 OD at 450 nm = 3.94 ± 0.15 g dry mycelial weight.

Conflicts of interest

Authors declare no conflict of interest.

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