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Original Article

Identification of Limiting Factors for the Optimum Growth of *Fusarium oxysporum* in Liquid Medium

Shilpi Srivastava[1,2], Neelam Pathak[2], Prachi Srivastava[1]

[1]Amity Institute of Biotechnology, Amity University Uttar Pradesh (Lucknow Campus), Lucknow - 226 010, [2]Department of Biotechnology, Integral University, Lucknow - 201 126, Uttar Pradesh, India

ABSTRACT

Fusarium oxysporum is a highly ubiquitous species that infects a wide range of hosts causing various diseases such as vascular wilts, yellows, rots, and damping-off. Despite the immense economic significance of this phytopathogen, few workers have reported growth studies in this genus in submerged culture. In the present study, several parameters such as change in media pH, biomass, pattern of substrate utilization, viability of the fungal cells, and protein content were observed over a period of time. The fungal biomass increased at a slow rate for the initial 48 h and thereafter increased at an exponential rate. However, after about 8 days the rapid growth stabilized and the trend became more toward stationary phase. The concentration of glucose in the liquid media decreased rapidly up to the initial 4 days, followed by a slow decrease. The pH of the medium gradually decreased as the fungal growth progressed, the reduction being more pronounced in the initial 48 h. This study would be of immense importance for utilization of *F. oxysporum* for diverse applications because we can predict the growth pattern in the fungus and modulate its growth for human benefit.

Key words: Biomass production, *Fusarium*, glucose utilization, MTT assay, protein

INTRODUCTION

Fungi are an extremely versatile class of microorganisms that comprises mostly saprophytes thriving on dead organic material. A range of organisms are attacked by fungi that encompasses evolutionary distinct groups from lower to higher eukaryotes, most prominently plants, insects, and mammals, including humans. *Fusarium oxysporum* is a highly ubiquitous, anamorphic fungi that infects a wide range of hosts causing various diseases such as vascular wilt, yellows, corm rot, root rot, and damping-off.[1-3] It is an abundant and active saprophyte in soil and organic matter, with some specific forms that are pathogenic to vegetables, flowers, and field crops.[1,4] *F. oxysporum* has several specialized forms known as *formae specialis* (f.sp.) which are further divided into races, dependent upon the particular plant cultivars colonized.[5] The life cycle of *F. oxysporum* begins in the soil where the spore germ tube, or the mycelium, enters via wounds or penetrates the root tips of potential hosts. The developing mycelium enters the xylem vessels through their pits after traversing the root cortex and travels upward through the plant toward the stem and crown.[6] *F. oxysporum* produces three types of asexual spores, viz., microconidia, macroconidia, and chlamydospores.[7] Of these, microconidia are the most frequently produced, which are disseminated in the sap via the transpiration stream, germinating when their movement is impeded. This produces a significant negative impact on the water economy of the host plants due to

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Address for correspondence: Dr. Prachi Srivastava, Amity Institute of Biotechnology, Amity University Uttar Pradesh (Lucknow Campus), Gomti Nagar, Lucknow - 226 010, Uttar Pradesh, India. E-mail: p.srivastava2@gmail.com

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vessel clogging, resulting in severe wilting and eventually death.[6] The fungus then invades the parenchymatous tissues and sporulates profusely, with the spores being disseminated subsequently via wind and rain.[8]

Despite the immense economic significance of this phytopathogen, few workers have reported growth studies in this genus in submerged culture.[9,10] Also, the changes induced by the fungus in the culture such as change in pH and the pattern of utilization of the substrate have also not been worked upon. Similarly, most of the researchers have evaluated the fungal protein content in growth media and natural environment,[11,12] but changes in other growth parameters over a period of time have been largely overlooked. Therefore, the present investigation was conducted to study the pattern of changes on the growth of the fungus for its utilization in diverse fields.

MATERIALS AND METHODS

Fungus

The fungus (*F. oxysporum* NCIM No. 1072) was obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune. Culture of fungus was done in petri plates on solid media, viz. potato dextrose agar (PDA) medium and kanamycin was used as an antimicrobial agent. All stock cultures were maintained on potato dextrose agar slants and subcultured every 1 month. The slants were incubated at 25°C for 7 days and then stored at 4°C.

Inocula preparation

A part of the fungal colony was then transferred into the potato dextrose broth by aseptically punching out 5 mm of the agar plate culture with a cutter. A shake flask culture was carried out in several 250-ml flasks containing 50 ml of the medium at 130 rpm and incubated at room temperature over a period of time. The inoculum was periodically tested at every 24 h for various growth parameters as provided below.

Dry weight of the fungus/biomass production

The fungal mycelium was harvested after every 24 h of growth, separated from the culture liquid by filtration through a Whatman No. 1 filter paper. The mycelial pellet was repeatedly washed with distilled water and dried at 70°C overnight. The dry weight of the fungus was calculated using the following formula:

Dry weight = (weight of filter paper + mycelium) - (weight of filter paper)

The standard curve was prepared using the data collected above.

Glucose utilization

Glucose utilization was periodically measured using the DNS reagent method as suggested by Miller.[13]

MTT assay

The MTT assay was performed as suggested by Denizot and Lang[14] and later modified by Freimoser *et al*.[15] An MTT stock solution (5 mg of MTT/ml of distilled water) was filter sterilized and kept at 4°C. To start the reaction, stock solution was added to growing cultures having a final concentration of 0.5 mg/ml. The mixture was incubated for 16 h on a shaker (160 rpm at 20°C). Cells were pelleted by centrifugation in Eppendorf tubes (15,000 *g*, 5 min), and the medium was removed and 500 ml of 1-propanol was added to the cells, and the tubes were vortexed. Lysed cells and debris were pelleted (15,000 *g*, 5 min), and 100 ml of the supernatant was transferred into a 96-well plate. The OD was measured with a spectrophotometer at 560 nm. A blank with propanol alone was measured and subtracted from all values.

pH variation over time

The pH of the culture filtrate was taken at every 24 h using a pH meter. A standard curve was prepared for the pH of culture filtrate over time.

Protein estimation

Total protein contents in the pellet and supernatant were determined by the method of Lowry *et al.*[16] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Dry weight of the fungus/biomass production

Productivity in terms of biomass production determines the fitness of organisms and can be sometimes used as a surrogate for organism activity in an ecosystem or media. Saprophytic fungi grow on complex organic compounds and render them into simple forms. This results in the production of a large amount of fungal biomass. Mycelia yield vary widely depending upon organisms and substrates. The changes in biomass of *F. oxysporum* over a period of time have been provided in Figure 1. The fungal biomass increased at a slow rate till about 48 h but thereafter increased at an exponential rate. However, after about 8 days this rapid growth stabilized and the trend became more toward stationary phase. Such a growth pattern has also been earlier described for *Fusarium verticillioides* strains wherein the strains showed a lag phase up to 24 h of culture and subsequent logarithmic phase.[17] The lag phase up to 24 h of culture is usually observed when fresh medium is inoculated with cells derived from an old culture. In such a scenario, the cells

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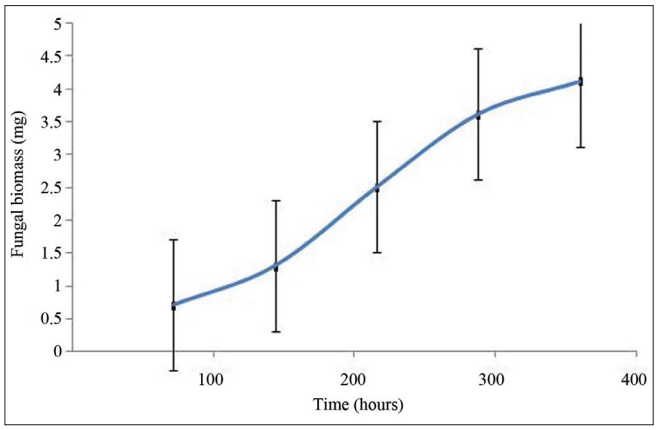


Figure 1: Change in fungal biomass over time (bars represent standard error values at 5% significance)

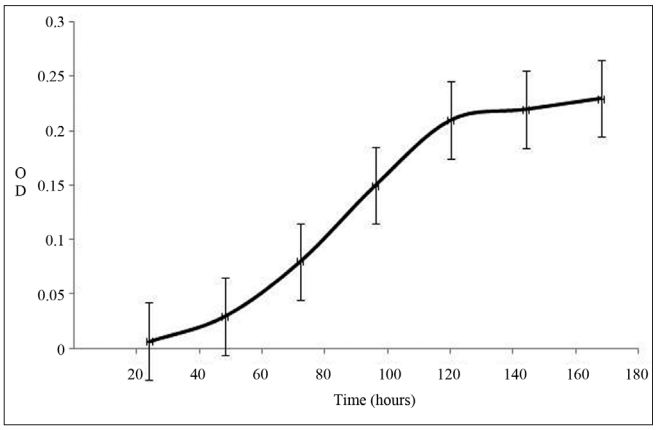


Figure 2: MTT assay showing OD vs. time (bars represent standard error values at 5% significance)

are deprived of enzymes and the total growth rate can only be reached when the optimum concentrations of these substances for synthesis are restored.[17] Several workers have also reported an initial lag period, followed by rapid growth in diverse fungal genus such as *Piromyces*.[18]

MTT assay

The colorimetric method for the determination of cell densities using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is more accurate and timesaving. The respiratory chain and other electron transport systems reduce MTT and other tetrazolium salts and form non-water-soluble violet formazan crystals within the cell. The amount of these crystals can be determined spectrophotometrically and serves as the number of living cells in the sample. The reaction occurs only in living cells and the results with dead cells are almost completely negative.[19] The tetrazolium salt MTT has been used for viability testing of various yeasts and filamentous fungi.[15,20,21]

The MTT assay in this study showed a gradual increase in the number of living cells over a period of time [Figure 2]. The pattern shown here is somewhat similar to that exhibited in the figure showing increase in the fungal biomass as time progressed. This increase in the number of living cells continued rapidly in the initial 5 days and thereafter showed a slow increase.

Glucose utilization

The usage and availability of growth substrate such as glucose is of immense importance to predict the growth rate and multiplication of fungi in laboratory cultures. This is more so if we want a good and luxuriant growth of fungi for industrial purposes. The concentration of glucose in the liquid media containing *F. oxysporum* decreased rapidly upto the initial 4 days, followed by a slow decrease [Figure 3]. Such a trend of high glucose usage in the initial growth period has also been observed in other microbes.[22-24] The high residual glucose concentration at the initial growth phase was attributed to increasing activity of starch hydrolyzing enzymes such as amylases and pectinase which convert the substrate to simple sugar that was utilized by the fungus. However, once the carbohydrates were utilized, growth slowed down because the fungus afterwards mainly relied on the utilization of its metabolic end products for growth.

pH variation over time

Figure 4 presents the changes in pH of the growth medium as fungal growth continues over a period of time. The results show that the pH of the medium gradually decreases as the fungal growth progresses. However, the reduction is more in the initial 48 h; thereafter the reduction in pH slows down. Such a reduction in the pH of the media has also been observed in other fungus such as *Glomus intraradices*,[25] wherein a decrease in the pH of the medium was observed on extensive mycelium development. This reduction in pH may be due to end products of metabolism that are secreted in the medium by the fungus, especially carbon dioxide. The pH variation seems to be inversely proportional to fungal growth because a lowering in pH resulted in higher fungal growth. Our results are similar to that observed in other fungi such as *Rhizopus*,[26] wherein the growth of the fungus reduced as the pH increased until none was recorded beyond pH 6.0. A consistent high mycelia length was recorded at low pH and the fungal growth rate reduced as the pH increased toward a neutral range until growth was not supported at pH 7.0 and beyond.[26]

Protein estimation

Filamentous fungi are widely used in industry to produce small-molecule metabolites such as antibiotics, cholesterol-lowering drugs, and food ingredients. Filamentous fungi are also unique in that they are adapted to produce and secrete proteins because of their biological niche

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as microbial scavengers. The natural ability of fungi to produce proteins has been widely exploited, particularly in the production of industrial enzymes. While the levels of protein production in natural isolates are generally not high enough for commercial exploitation, culture modulation can lead to enormous yield increases, making it possible to produce large amounts of protein that can be commercially exploited. In estimating the true protein content of biological material, it is important that the method of protein determination is chosen which is accurate and convenient enough to be used for routine testing. The Folin method of protein determination, applied to fungal biomass,[12] can be reliable and convenient for routine determinations of the true protein content. The protein content in the pellet was about 24.6 mg/g after the initial 24 h of growth which is quite comparable to that reported for other fungi.[27] During the subsequent period of nitrogen depletion, there was a very marked and persistent decrease in the overall amount of protein. The protein content of the fungus decreased to 112 mg/g after about 6 days of culture, a decrease of more than 50% [Figure 5a]. The same trend was observed in the protein content of the supernatant which had considerable low protein (138 mg/g) when compared with the pellet [Figure 5b]. However, the decrease in protein content was remarkable because a decrease of 66% was observed after 6 days of culture. Thus, under conditions of nitrogen starvation, there was a rapid and specific loss of those proteins, a fact observed in other fungi such as *Penicillium griseofulvum*.[28]

Rapidly increasing world population has resulted in a rising demand of protein for both human and animal consumption. The situation has intensified more due to the escalating prices of traditional protein ingredients.[29,30] There is an urgent requirement for new sources of protein that will not require agricultural land, costly and tedious means of production. Microbial proteins are microbial cells grown and harvested for use as a protein source for human and animal consumption.[31,32] This microbial protein is referred to as a whole microbial biomass which can be derived from a variety of microorganisms both unicellular and multicellular, namely bacteria, yeast, fungi, and microscopic algae. A number of advantages such as rapid succession of generations, easy genetic modification,

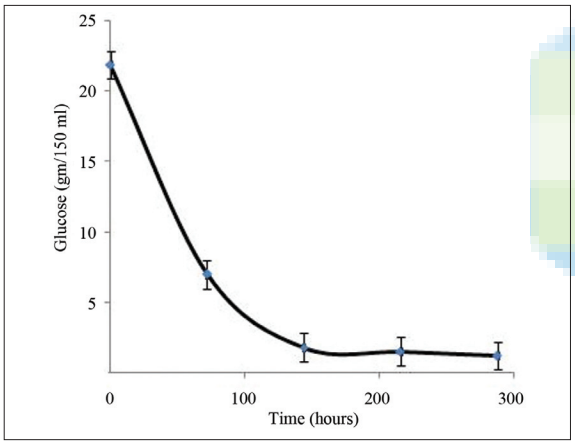


Figure 3: Glucose utilization by the fungus over a period of time (bars represent standard error values at 5% significance)

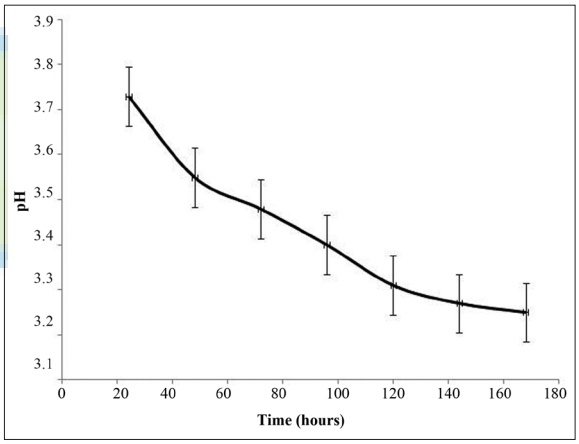


Figure 4: Graph depicting pH of culture filtrate at different time intervals (bars represent standard error values at 5% significance)

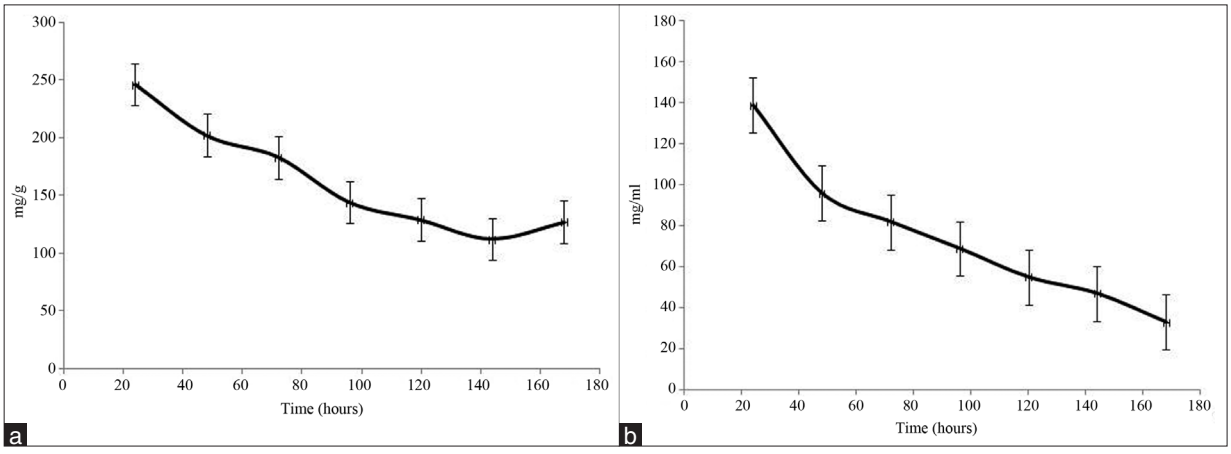


Figure 5: (a) Variation in protein content in the pellet over time (bars represent standard error values at 5% significance) (b) Variation in protein content in the supernatant over time (bars represent standard error values at 5% significance)

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high protein content, broad spectrum of raw material used for the production, production in continuous cultures, consistent quality, no land requirements and easy regulation of environmental, and growth factors.[33] According to the literature, *F. oxysporum*, along with two other species, viz., *Fusarium graminearum* and *Fusarium solani*, are considered edible strains of *Fusarium*.[34] The value for protein content obtained in this study agreed with the protein content value obtained in the study on *F. oxysporum* by Christias *et al*.[12] However, the protein values of this study were slightly less than the values obtained in *F. oxysporum* in a recent study,[35] wherein the fungus was explored for production of mycoprotein. The lower protein content obtained in this study was probably due to the use of different growth media in both the experiments. The amino acid composition of *F. oxysporum* mycoprotein is also comparable with that of the soybean meal and FAO reference protein.[35] Thus, *Fusarium* seems to be a good organism for microbial protein production. Preliminary experiments have shown that *Fusarium* spp. grow well and produce satisfactory yields of biomass in liquid cultures. Also, *F. oxysporum* is also reported to contain high amounts of all essential amino acids and grows well giving satisfactory yields of biomass in liquid shake cultures utilizing inexpensive agricultural waste products.[12] Therefore, *Fusarium* is a much better organism for microbial protein production than other fungi such as *Aspergillus* and *Penicillum*. More efforts are needed to explore the possibilities of *Fusarium* as a protein source.

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