Original Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

EFFECT OF AGITATION ON MYCOPROTEIN PRODUCTION AND ITS INFLUENCE ON MASS TRANSFER COEFFICIENT AND A STUDY ON THE POTENTIAL OF MYCOPROTEIN AS ANTITUMOR AGENT

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ABSTRACT

Fusarium venenatum has been used as a source of mycoprotein for animal and human consumption in European countries as it is rich in protein, carbohydrates, essential amino acids and less fat content. In this study, *Fusarium venenatum* (CBS 458.93) was utilized for the production of mycoprotein in shake flask, 2 L, 20 L and 75 L fermenters. The effect of agitation and its influence on mass transfer coefficient were studied. The production of biomass was found to be 4.6 gm in 5 L shake flask, 5.4 gm in 2 L fermenter, 5.8gm in 20 L fermenter, and 6.3gm in 75 L fermenter. The fungal protein was checked for antitumour activity against MCF cell lines by MTT assay. The antitumor potential was found to be significant as the IC_{50} value was found to be 29.5 µg/ml. The toxicity was not very significant against normal Vero cells as the IC_{50} values was not found in the tested concentration thereby making it a potential candidate in cancer management.

KEYWORDS: ***Fusarium venenatum, Mycoprotein, Agitation rate, mass transfer coefficient, anticancer activity***

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Received on: 10-01-2017
Revised and Accepted on: 05-05-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.3.b49-54

INTRODUCTION

Microorganisms are an excellent source of single-cell protein (SCP) which is described as the protein-rich cell mass derived from microorganisms. It is grown on a large scale for either animal or human consumption as SCP has a high content of protein containing all the essential amino acids. Microorganisms are suitable for SCP production because of their rapid growth rate, their ability to use inexpensive raw materials, and high efficiency, expressed as grams of protein produced per kilogram of raw material. In spite of these advantages, only one SCP product approved for human consumption has reached the market which is the mycoprotein obtained from *Fusarium venenatum*. One genus of filamentous fungi *Fusarium* (Latin word fusus meaning spindle) also referred to as hyphomycetes is widely distributed in soil and associated with plants. Most of the species are harmless saprobes and are found to be relatively abundant members of the soil microbial community. *Fusarium venenatum* is a microfungus that was discovered in Buckinghamshire in the United Kingdom in 1967 by ICI as part of the effort find alternative sources of food to fill the protein gap caused by the growing world population.[1] This fungus is found to contain high protein content and hence used commercially for the production of single cell protein mycoprotein.Large-scale production of mycoprotein involves the strain to be cultured in viable environmental, economical and optimized conditions like pH, temperature and process parameters such as aeration and agitation. Optimization is targeted to maximize the production of the final product with compliant manufacturing processes. Large scale cell cultivation for the product is performed in specialized reaction vessels known as bioreactors or fermenters. The fermentation process must be developed parallel with the purification process as they affect each other. This remains a crucial feature for the success of the scaling up process.[2] The dissolved oxygen content is known to influence the metabolism of the fungi and there by its growth. The increase in specific oxygen uptake rate is dependent on the dissolved oxygen concentration upto a certain level (C_{crit}) atfter which oxygen uptake remains constant. Hence, biomass production can be increased by fulfilling the organisms specific oxygen demand as the metabolism of the organism would be disturbed if the oxygen concentration is less than the essential level. this context, a study was carried out to produce maximum biomass of *Fusarium venenatum* by varying the agitation and finding its effect on the mass transfer coefficient. The biocompatibility of the biomass was checked against (Vero cells) and also its ability to induce toxicity in against cancer cells (MCF 7 cells) was assessed as breast cancer is the most common tumor among women and still remains the leading cause of cancer mortality despite extensive prognosis and survival.

MATERIALS AND METHODS

Fusarium venenatum (CBS 458.93) obtained from Netherland (Fungal Biodiversity Centre), was initially used for the production of mycoprotein as described elsewhere.[3]

Optimization of RPM and Determination of volumetric mass transfer coefficient (k_La)

This study tried to optimize the rpm in shake flask at 100 rpm to 150 rpm, 2 L bench scale fermenter (Sartorius) at 150, 200 and 250 rpm, 20 L fermenter (Lark) at 250, 300 and 350 rpm and 75 L fermenter (Scigenics) at 350 rpm. After 72 hr the biomass was harvested and the k_La was determined by dynamic gassing out method.The determination of k_La of a fermenter is essential in order to establish its aeration efficiency and to quantity the effects of operating variables on the provision of oxygen. Though a number of methods like sulphite oxidation technique, gassing out techniques (static and dynamic) are available, dynamic gassing out technique is widely employed industrially.[4] 2L, 20 L and 75L fermenter was aerated through a compressor. Air was passed into the fermenter through a flow meter/regulating valve system (to regulate the flow rate), then a sterilizing filter. It was introduced at the bottom of the filter through a sparger, which is an arrangement of pipe work perforated with small holes. By shutting off the regulating valve, the air supply to the fermenter was stopped. The decrease in the dissolved oxygen concentration was noted down at regular intervals of 10 seconds. After the dissolved oxygen (DO) level came to around 10%, the valve was then opened to allow the inflow of air. The rise in DO concentration was noted at regular intervals of 10 seconds till the value became constant. The rate of oxygen transfer from air bubble to the liquid phase may be described by the equation: $dC_{AL}/dt = k_La(C^*- C_{AL})$-(1)

Extraction of Mycoprotein

Fusarium venenatum was cultivated in a medium that contained date extract, jaggery water, KH_2PO_4, K_2HPO_4, $MgSO_4$. The inoculated medium was incubated at 28°C for 10 days. After incubation, the broth was centrifuged at 10,000 rpm for 10 min. The supernatant was collected and extracted with Methanol (1:1) separately and concentrated using rotary evaporator. The dried extract was scraped and resuspended in DMSO and used to perform cytotoxicity studies.

Cytotoxic of the mycoprotein

Vero and MCF 7 cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India and were maintained at 27°C using Grace's TNM-FH medium supplemented with 10% FCS and 1% penicillin–streptomycin solution. Cells were collected six days after sub culturing and diluted with fresh medium to a density of 7.5 x 10^4 cells/ml. Each well of a 96-well microtiter culture plate was loaded with 100 µl of cell solution containing 2 µl of the fungal metabolite, prepared in DMSO. The final concentrations of fungal metabolites were 3.95, 7.8, 15.6, 31.2, 62.5, 125, 250 µg/ml.[5] The test medium was replaced with 20 µl of 2 mg/ml MTT followed by overnight staining at 27 ºC. The staining solution was carefully removed and DMSO (150 µl/well) was added to solubilize the purple formazan crystals produced within the cell. The absorbance of each well was measured at 540 nm using a microplate reader.[6] The cell growth was expressed as percentage of absorbance ratio (absorbance in wells with fungal metabolites to control well without fungal metabolites).

RESULTS AND DISCUSSION

Our previous studies have reported the production on mycoprotein from *Fusarium venenatum* by optimizing the media components using statistical methods namely Plackett Burman model and Response surface methodology.[3,7] This study aims to find the effect of agitation on the growth and also tries to assess its toxicity.

Medium and conditions for fermenter

After optimizing the nutritional parameters, the fungus was grown in this optimized medium in fermenters of capacitates 2 L, 20 L and 75 L. Table 1 provides the details on the physical parameters that were adopted for the cultivation of the fungi on a large scale.

Table 1
Medium components and conditions employed in fermenters

Components	250 ml Flask	2 L	20 L	75 L
Liquid Jaggery (ml/l)	10	10 * 1.5	10 * 13	10* 35
Date extract (ml/l)	80	80 * 1.5	80*13	80 * 35
K_2HPO_4 (mg/l)	500	500 * 1.5	500 *13	500 *35
KH_2PO_4 (mg/l)	500	500 *1.5	500 * 13	500* 35
Inoculum size (%)	5	75	650	1750
PHYSICAL PARAMETERS				
Working volume	100 ml	1.5 L	13 L	35 L
Initial pH	6.0±0.2	6.0±0.2	6.0±0.2	5.9±0.2
Final pH	5.3	5.3	5.4	5.5
Temperature	32°C ± 2	33°C ± 2	33°C ± 2	33°C ± 2
Agitation (rpm)	130	200	250	350
Aeration (vvm)	-	0.3-0.5	0.3-0.5	0.3-0.5

Optimization of rpm in shake flask and fermenters and its effect on k_La

Studies were carried out to determine the optimal agitation for the enhanced production of biomass in the fermenter. Agitation is a crucial parameter in a fermentation process as this ensures proper mixing of the nutrients inside the fermenter thereby providing a homogenous environment. It also helps in efficient aeration and heat transfer inside the fermenter. Inadequate oxygen supply may lead to changes in the enzyme system or may sometimes lead to the death of the organism subsequently resulting in lower yield of the product .[8,9] Agitation generates shear forces that causes morphological changes in the organism, produces variation in the growth and influences the product formation and sometimes may lead to cell damage.[10,11] Lower agitation speed may lead to insufficient oxygen supply in the fermenter while higher agitation may reduce the enzyme production.[12] Therefore, it is essential to determine the optimal agitation speed in order to obtain maximal biomass.Volumetric mass transfer coefficient (k_La) provides rate of oxygen transfer which is dependent upon the dissolved oxygen (DO) concentration C_L and the oxygen saturation concentration in the liquid phase at the gas-liquid interface.[13,14] k_La has been extensively studied as a crucial parameter for bioreactor performance and is considered to be the common criteria for scale-up along with power to volume ratio in a gassed fermenter. This is dependent on a number of factors like agitation, air-flow rate, and medium and culture rheology. k_La is measured by dynamic gassing-out method which is most commonly used where in there is short period of gas out or non-aeration and subsequent gas in or re-aeration during which the dissolved oxygen concentration is monitored.[15]

Table 2
RPM maintained and the biomass produced along with k_La values

TRIAL	RPM	BIOMASS g/l	k_La (S^{-1})
FLASK	100	3.4	-
	120	3.9	-
	130	4.6	-
	140	4.4	-
	150	4.3	-
2 L	200	4.6	0.0160
	250	5.4	0.0250
	300	5.1	0.0150
20 L	250	5.3	0.0085
	300	5.8	0.0250
	350	5.2	0.0125
75 L	350	6.3	0.0047

The present study investigates the effect of agitation on the production of the biomass in three volumes of reactors along with shake flask culture and the results are provided in table 2. In shake flask culture the maximum biomass production was obtained at 130 rpm which was 4.6 g/L. The production of biomass in 2 L fermenter was found to be high at 250 rpm with 5.4 g/l while in 20 L and 75 L the maximum production was found at 300 rpm and 350 rpm with 5.8 g/l and 6.3 g/l biomass, respectively. Adequate levels of oxygen must be provided to the liquid broth for the microbial growth. Because oxygen is sparingly soluble, the microbial

processes become oxygen limited. At times, oxygen transfer rate becomes a limiting factor of a bioreactor set up and hence remains an important parameter for scale up. A fermentation medium under saturated conditions contains approximately 7.6 mg/l of oxygen at 30°C. If unaerated, this lasts for barely 30 seconds as it rapidly metabolized by the active culture The yield obtained at different agitations is shown in Table 2 for 3L, 20L, and 75L fermenters. It has been observed that the change in agitation affects the substrate conversion (i.e.) at lower speed, the uptake of substrate is found to be very low, which is mainly due to the incomplete mixing or lower mass transfer resistance. This was identified by calculating the mass transfer resistance at various speeds. The mass transfer coefficient (k_La) values were determined at 3 different speeds 200 rpm (0.016 S^{-1}), 250 rpm (k_La 0.025 S^{-1}), and 300 rpm (k_La 0.015 S^{-1}) in 2 lit fermenter. The maximum biomass (5.4g/l) was found at 250 rpm where the mass transfer coefficient (k_La) value was 0.025 S^{-1}. Similarly the k_La value was obtained in a 20 lit fermenter at various agitations (250 rpm, 300 rpm and 350 rpm) and was found to be k_La 0.0085 S^{-1}, k_La 0.025S-1 and k_La 0.0125 S-1 respectively. Among them maximum biomass was obtained at 300 rpm (5.8 g/l). In 75 lit fermenter at 350 rpm, the k_La value was 0.00475S^{-1}.

Toxicity studies

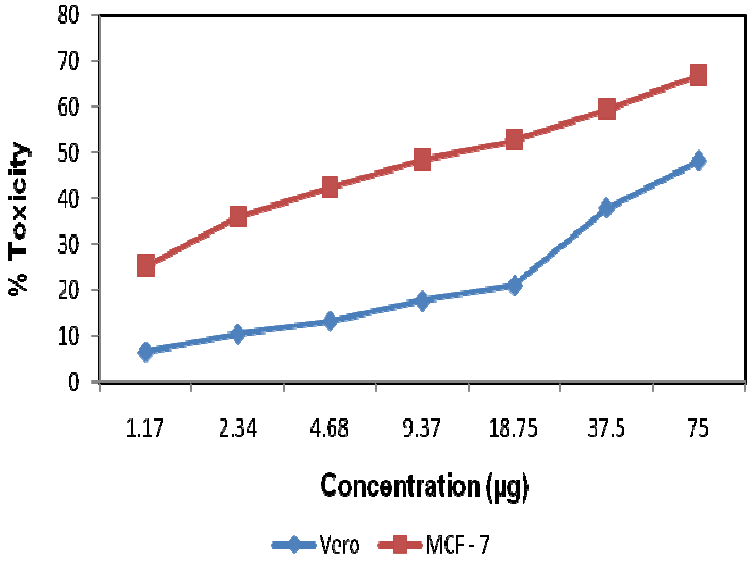
The capability of living species in producing varied classes of bio active compounds in the form of secondary metabolites is well established. Studies have shown that fungal strains are an exceptional source of these secondary metabolites with potent bioactivities. Reports have suggested that over 40% of medicines which originated from natural sources are produced from fungal metabolites.[16] In view of the promise displayed by fungi as a source of various medicinally important compounds, the present study has been undertaken to assess the anticancer potential of *Fusarium venenatum* extracts against cancer cells (MCF 7 cells) along with its compatibility with normal cells (Vero cells) and the results are provided in Table 3.

Table 3

Cytotoxicity of the Fusarium venenatum extracts on MCF – 7 cells

Concentration of *F. venenatum* extract (µg)	% Cytotoxicity	
	Vero	MCF - 7
1.17	6.5	25.2
2.34	10.46	36.1
4.68	13.25	42.5
9.37	17.65	48.4
18.75	20.98	52.8
37.5	37.95	59.5
75	48.20	67

It has been observed that as the concentration of the extract increased there is a corresponding increase in the cytotoxicity in both the cell lines (Fig 1). The IC_{50} value was found to be 12.2 µg/ml for MCF 7 cells while the IC_{50} value was not found in Vero cells for the tested concentration These results indicates that a higher concentration was required to initiate apoptosis in normal cells while a lower concentration of 29.5 µg/ml was found to be sufficient to induce toxicity in cancer cell lines.



Microscopic images of MCF 7

Figure 1

Percentage of Cytotoxicity of methanol extracts on Vero and MCF cell lines

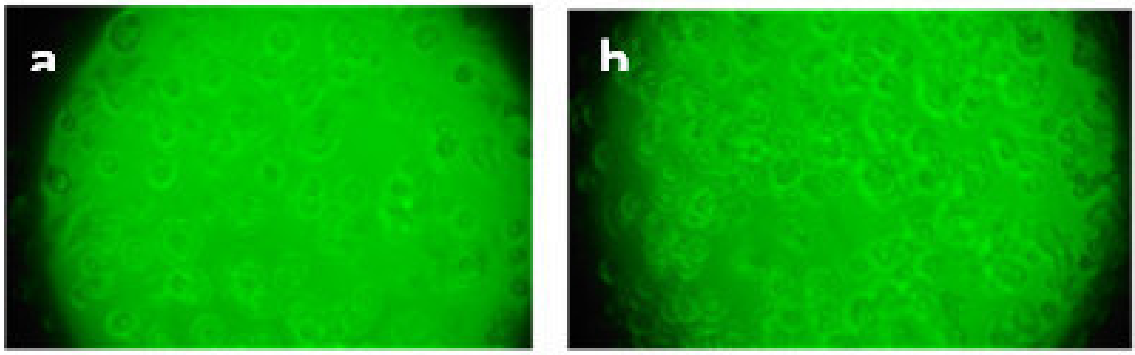


Figure 2
Microscopic images(40x) of MCF 7 cells after exposure to methanolic extract a) 0th hour b) 48 hours

The microscopic images provided in Fig 4 also showed a clear reduction in the number of cells thereby indicating the potential of the extracts of *Fusarium venenatum* in cancer treatment. These results are supported by the studies conducted by Kuriakose et al[17] who reported that an endophytic sp *Fusarium solani* was effective in producing metabolites that showed toxicity to five human cancer cell lines namely HepG2, HeLa, MCF-7, OVCAR-3 and PC-3. They observed that this toxicity was brought about by inducing apoptosis through the mitochondrial pathway which was indicated in the loss in membrane potential of mitochondria, fragmentation of DNA and condensation of nuclear chromatin. Furthermore, many of the endophytic fungi belonging to the class *Fusarium* are known to produce potential bioactive compounds.[18] Wu reported that the metabolites produced by *Morinda citrifolia* L. leaf isolated endophytes inhibited the growth of LU-1 (lung), PC-3 (prostate) and MCF-7 (breast) cells where the IC_{50} values was found to be ≤10 μg/Ml.[19] *Aspergillus* sp was also identified as a potential source of anticancer agents as this was found to show promising toxicity to HeLa cells and MCF 7 cells when compared with Cisplatin. The mechanism of toxicity was found to be apoptosis identified by accumulation of cells in Sub G0 phase as revealed by flow cytometric analysis along with cell necrosis.[20] Similarly methonolic extract of fruiting bodies of *Calocybe indica* Var APK2 also showed noticeable apoptotic activity against A549 lung cancer cell lines there by advocating the potential of mushroom as a source of anticancer compounds.[21] Another study reported toxicity of polysaccharides isolated from *Pleurotus sajor-caju* and *Lactuca Sativa against* colon (HCT 116), liver (HEPG2), cervical (HELA) and breast (MCF7) carcinoma cell lines. [22] Another study reported that the ethanol extracts of the fungal sp *F. pinicola* sarcoma 180 tumors. This was evaluated by the growth inhibitory ratio (82.8%, $p < 0.001$) thereby increasing cell apoptosis and also tumor growth inhibitory ratio (54%, $p < 0.05$) that reduced the size of the tumor. These in vitro and in vivo studies revealed the promise of the fungi in bringing about toxicity to cancer cells and therefore can be a potential candidate for various biomedical applications.[23]

CONCLUSION

The nutritional value of Fusarium venenatum in terms of its richness in protein, carbohydrates, essential aninoacids and less fat made it a food source for human and animal consumption in European countries. In this context, scale up of Fusarium venenatum growth was carried out in different capacity fermentors and study the influence of agitation rate on the production. The significance of agitation rate on the growth of *Fusarium venenatum* has been demonstrated on a large scale in the present study. The agitation rates were found to influence the biomass production which was found to be to be 4.6 gm in 5 L shake flask, 5.4 gm in 2 L fermenter, 5.8gm in 20 L fermenter, and 6.3gm in 75 L fermenter. Additionally, the methanol extract of *Fusarium venenatum* was used to evaluate its anticancer potential against MCF-7 cell line. It was observed that the extract displayed noticeable cytotoxicity against MCF 7 with IC_{50} values being 12.2 µg/ml. This study reveals the potential of *Fusarium venenatum* as synthesizer of secondary metabolites with significant bioactivities.

CONFLICT OF INTEREST

Conflict of interest declared none.

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We sincerely thank the above reviewers for peer reviewing the manuscript