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# Effect of Different Factors on Fermentative Production of Enzymes by Fungi

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## ABSTRACT

Fungi are exploited to produce extracellular enzymes which are of prime importance in academia, industrial biotechnology and ultimately commerce. To produce and exploit enzymes during the fermentation processes by fungi, various factors can affect productivity. The environment provided to the fungi for such tasks should be friendly, and henceforth, the environment where the fungi are to be cultivated must provide uniform distribution of nutrients, maintenance of optima hydrogen ion concentration, temperature, oxygen through agitation where the last mechanical factor could cause the fragmentation and breakage of the fungal mycelia thus influencing the yield.

**Keywords:** aeration, enzyme, mechanical force, media composition, morphology, pH, temperature, yield

**Abbreviations:** **α**, the angle of blade; **D/T**, the ratios of impeller diameter to vessel diameter; **g**, generation time; $\mathbf{K_La}$, volumetric oxygen transfer rate; **n**, number of blades; $\mathbf{t_d}$, doubling time; **μ**, specific growth rate; **W/D**, height of the blade to impeller diameter; $\mathbf{Y_{P/S,}}$ product yield coefficient; $\mathbf{Y_{X/S}}$, growth yield coefficient(g biomass/g substrate)

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## INTRODUCTION

Enzymes are biomolecules produced by living cells which bring about biochemical reactions within or outside of cells leading to the formation of energy or new cellular components. An organism can be considered a factory where thousands of reactions are led by enzymes taking place in or outside of the cell. Therefore, the entity of the cell is governed by the enzymes and this makes them of prime importance. Man has understood this importance since he learnt how to make bread, cheese, vinegar, alcohol, nowadays fine chemicals and therapeutic agents. Enzymes have found many applications in biotechnological processes leading to the synthesis, transformation of compounds to fine chemicals, semi-synthetic antibiotics (Dariush *et al*. 2002, 2003, 2007a, 2007b, 2007c), and biosensors (De *et al*. 2004).

In this review article, the author intends to emphasize the importance of the factors affecting the fermentation process leading to the production of enzymes by microorganisms, particularly fungi. Fungi with the development of industrial biotechnology have been exploited as useful tools and are considered the main source of enzymes and other metabolite production. The factors which are considered to influence such fermentation processes are as follows: nutritional requirements, temperature, pH, mechanical force, aeration and morphology of the fungi which may depend upon such factors.

## NUTRITIONAL REQUIREMENTS

Fungi require nutrients for their growth and metabolite activity and this can be provided to the organisms in the form of simple or complex media.

The media employed in fungal submerged cultivation can favor growth as well as product formation. Fungi relatively need elements supplied by nutrients such as oxygen, hydrogen, C, N, P, K, S, Mg, Fe, Zn, Cu, the first four considered to be in macro and the rest in micro quantities, i.e. the macro- and micronutrients, respectively. The media used in fermentation processes can either be synthetic or complex. Complex media are often used in enzyme and antibiotic production (Chisti 1999). The fungal cell needs carbonaceous materials as a carbon source to maintain and develop its dynamic state. The fate of carbonaceous materials during the cell's cycle can be considered simple or complex. However, part of such material is converted to cellular components, especially the cell wall, some along with nitrogenous components to proteinacious, nuclear materials and other organic acids; the latter causes the inhibition of growth and finally low product recovery. Papagianni *et al*. (1999) studied the submerged fermentation of phytase by *Aspergillus niger*. The addition of undissolved organic complex carbohydrate (wheat bran) to the medium enhanced growth and enzyme production. This was related to the continuous availability of P supplied through the hydrolysis of wheat bran by phytase where such an element was provi-

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Invited Mini-Review

**Table 1** Effect of different carbon sources on enzyme fermentation

| Carbon sources | Organisms | Enzymes | References |
|---|---|---|---|
| Wheat bran | *Trichoderma viride* | β-Glucosidase | Ikram-ul-H *et al*. 2006 |
| Malto-dextrin | *Thermomyces lanuginosus* ATCC 34626 | α-Amylase | Quang *et al*. 2000 |
| Dextrin | *Thermomyces lanuginosus* ATCC 34626 | Glucoamylase | Quang *et al*. 2000 |
| Lactose | *Aspergillus ochraceus* | α-Amylase | Nahas *et al*. 2002 |
| Soluble starch | *Candida famata* | α-Amylase | Lagzouli *et al*. 2007 |
| Cassava starch | *Trichoderma* spp. | Glucoamylase | Chavez *et al*. 2004 |
| Maltose | *Trichoderma* spp. | α-Amylase | Chavez *et al*. 2004 |

ded to the organism. On the contrary, an increase in P concentration shifted metabolic overproduction to over growth of the organism during xylanase production by *Aspergillus awamori*, which was characterized by an increase in specific growth rate (μ), other metabolites and unwanted morphology (Schügerl *et al*. 1998). Clado *et al*. (2002) studied the production of cutinase by a recombinant unicellular fungus, *Saccharomyces cerevisiae*. They used glucose and galactose as carbon sources to compare the synthesis of cutinase in pre-fermentation processes. Glucose (2%) as a carbon source could yield more cutinase when employed in a chemically defined medium in the pre-fermentation process but the yield was higher in complex medium containing galactose (2%) as a carbon source. One of the most important parameters in fermentation process is the level of metabolizable substrates used. In the presence of a readily metabolized carbon source like glucose many fermentation processes can be repressed (Johri *et al*. 1990; Ates *et al*. 1997). Therefore, glucose can inhibit the growth of the organism by a repression effect and thus leads to poor growth. It is essential to use an optimum level of this carbon source or plan another fermentation process strategy like fed batch or continuous production depending on the organism in relation to product formation. In this sense, four different glucose concentrations varying from 1 to 10 g $l^{-1}$ were employed to observe the effect of such a concentration range on lipolytic activity of immobilized *Rhizopus arrhizus*. The maximum lipase production by such a fungus was obtained at a glucose concentration of 1 and 2.5 g/L and any further increase in glucose concentration decreased lipase production (Elibol and Ozer 2000). In industrial biotechnology, the media composition is critical in the sense that the composition of the medium affects the product concentration, yield and volumetric productivity. The importance of the composition of the medium is from the point of its economy (Souza *et al*. 2006). Macedo *et al*. (2007) studied the production of transglutaminase, another important enzyme in food industries (Zhu *et al*. 1995; Kawan and Easa 2003) by pseudo-fungus *Streptomyces* sp. through medium optimization. Macedo *et al*. found that peptone (2%) and a mixture of potato starch (2%), glucose (0.2%) and maltodextrin (2%) alone as nitrogen and carbon sources yielded 1.12, 1.12 and 1.18 U $ml^{-1}$ transglutaminase, respectively. Since peptone is more expensive than other nitrogen sources like soybean flour (2%), it was replaced by soybean flour which adversely affected enzyme production. Optimization of medium components for the production of another industrially important enzyme, lipase, by *Aspergillus carneus* indicated glucose at 0.8% (w/v) to be optimal in yielding a high titer of lipase (Kaushik *et al*. 2006). The final yield of 12.7 U $ml^{-1}$ of lipase was obtained under optimal medium components of sunflower oil (1%), glucose (0.8%), and peptone (0.8%). Production of lipases by fungi is mostly inductive and different inducers have been used to induce lipase production by fungi viz., sunflower oil (Kaushik *et al*. 2006), corn oil (Elibol and Ozer 2000), olive oil (Valero *et al*. 1988) and Arabica gum (Ferrer and Sola 1992). Xylanase production by *Trichoderma harzianum* 1073 D3 in medium containing xylan, glucose, lactose, avicel, sucrose, maltose, carboxy methyl cellulose, peptone, ammonium sulphate, urea, ammonium nitrate and sodium nitrate individually as carbon and nitrogen sources. Xylan as a carbon source caused the production of xylanase after a maximum of 10 days' incubation (Isil and Nilufer 2005). Furthermore, when 0.2% glucose was incorporated into the medium containing xylan, the titer of xylanase reached 754.3 $Umg^{-1}$ protein within 7 days. This is related to the fact that glucose is readily hydrolyzed by the fungus thereby increasing the fungal biomass which in turn synthesizes the enzyme rapidly. In the above cited carbon sources, 0.2% sucrose with xylan gave 786.7 $Umg^{-1}$ protein of xylanase in 7 days. Ammonium sulphate (0.14%) as a nitrogen source increase the yield of enzyme and when this was added to the fermentation medium along with urea, a rise in production of xylanase could be observed (760.0. $Umg^{-1}$ protein) Effect of different carbon sources like glucose, sucrose, maltose, xylose, starch and cellulose on the production of xylanase by *Cochliobolus sativus* in submerged fermentation was reported to be low (Bakri *et al*. 2008) while in medium containing xylane the amount of xylanase reached a maximum (34.19 $IUml^{-1}$ after 120 h). This could be related to low molecular mass degradation products of xylan and cellulose hydrolysis penetrating into the cell and inducing the production of hydrolytic enzymes (Haltrich *et al*. 1996). **Table 1** shows effect of different carbon sources on enzyme fermentation by microorganisms.

## TEMPERATURE OF THE FERMENTATION PROCESS

The growth of fungi can be affected by the temperature of the fermentation process used to cultivate the fungi under cultivation leading to poor growth and ultimately yield. Henceforth, the temperature of the incubation during the fermentation process plays a crucial role to achieve good yield in accordance with growth of biomass; the cultivation temperature of the process depends on the fungus and the strategy to be followed. There are about three kinds of temperature cultivation modes for microbial growth in relation to fermentation yield/products; 1) fermentation at a single and constant temperature; 2) two-stage fermentation shift temperatures (Zheng *et al*. 2001); 3) oscillatory temperature (Zhang *et al*. 2002). The temperature of the fermentation process can affect the growth as well as the product yield of the fungi and pseudo fungi in close relation (Torja and Mas 2003). Temperature can affect the sensitivity of the yeast to product concentration, growth rate, rate of fermentation, viability, length of lag phase and membrane function (Jackson 2000). Sener and Canbas (2007) studied the effect of temperature on the growth of *Zymoflore* VL1 and *Saccharomyces cerevisiae*. Performing their studies by conducting experiments at 18 and 25°C for both organisms, they cautiously stated that no lag phase was observed during the growth of both yeast cells. The increase in temperature resulted in an increase in μ, $Y_{X/S}$ and a decrease in g, $t_d$, and $Y_{P/S}$ values. The microbial growth and conversion of carbon source to biomass increased with increasing temperature while the product yield ($Y_{P/S}$) decreased. In this regard, HE *et al*. (2004) studied the effect of temperature on the fermentative production of elastase. They performed their studies according to the above first strategy. They found that fermentation at constant 30°C was appropriate for the production of elastase by the *Bacillus* sp EL 31410. The two-stage shift and oscillatory temperature strategies did not improve biomass production and elastase biosynthesis compared to a single cultivation temperature. To indicate the importance of effect of temperature on the production of biological or non-biological materials by a fermentation pro-

**Table 2** Effect of pH on enzymes fermentation.

| Final pH | Organism | Enzymes | References |
|---|---|---|---|
| 7 | *Fusarium globulsum* | Alkaline lipase | Ruchi *et al*. 2005 |
| 5 | *Aspergillus ochraceous* | Amylase | Nahas and Waldemarin 2002 |
| 5 | *Candida famato* | Glucoamylase | Lagzouli *et al*. 2007 |
| 4.9 | *Thermomyces lanuginosus* ATCC 34626 | Amylase | Quang *et al*. 2000 |
| 6 | *Aspergillus flavus* A 1.1 | Amylase | Eleni *et al*. 2005 |

cess employing microorganisms, the steady state operation in chemical reactors can be employed to improve the performance of the reactors but this mode can not be applied to bioreactors due to the nature of the biological process. The unsteady state operation of reactors means that there must be changes in operational parameters such as temperature, pressure, concentration of fed substrate, flow rate, etc., in order to increase the productivity via externally forced methods. The unsteady state operation of temperature shift has been employed to increase the β-lactam antibiotic by 15% in penicillin fermentation (Constaninides *et al*. 1970 a, 1970b). Further, Yuan *et al*. (2005) investigated the temperature-dependent production of xylanase by *Aspergillus niger*. They observed higher temperature favored biomass growth but lowered xylanase production. An unsteady state operation with a fermentation temperature of 33°C for the first 24 hours and a subsequent drop of temperature to 27°C for the remaining of the fermentation process shortened the production process from 92 to 76 hours without an adverse effect on xylanase production. However, temperature can be considered as an environmental parameter that can be controlled during fungal cultivation. Temperature of cultivation will affect medium evaporation, especially in a continuous mode of fermentation. Medium evaporation causes errors in calculating the growth constant like Y and μ. This will happen at high temperature with low dilution rates in fungal fermentation (King *et al*. 1972).

## pH OF THE FERMENTATION PROCESS

Microorganisms grow, multiply and produce active proteins for their industrial and commercial exploitation when they are in a physio-friendly environment. One of the factors considered to affect their environment is pH of the medium where they are being cultivated. Therefore, pH of the medium can influence the growth of microorganisms largely leading to poor growth and production rate. Furthermore, changes in pH of the medium can shift the metabolites particularly when the targeted product is an organic acid and this may be due to control and balance of NADH, a key in the metabolic pathways employed by the cells in fermentation.

The effect of pH on β-glucosidase production by *Trichoderma viride* UVNG-4 was carried out by Ikram-ul-Haq *et al*. (2006). β-Glucosidase with 16.6 U $ml^{-1}$ $min^{-1}$ activity was produced with a protein content of 1.11 mg $mL^{-1}$ at pH 5.5, the optimum pH. More acidic and alkaline pHs did not support growth and enzyme production. Fungi are known to grow and secrete the desired proteins in acidic pH, e.g. *Fusarium globulosum* which produces lipase at neutral pH i.e. 7 even though the organism is capable of growing at pH ranging from 3 to 10 although the highest amount of lipase was secreted at pH 7 (Ruchi *et al*. 2005). The effect of pH of the medium on production of polygalacturonase (PG) by *Aspergillus niger* and *Penicillium dierckxii* over the pH values ranging from 2.0 to 8.0 was studied by Shubakov and Elkina (2002) who observed that maximum PG activity was produced by *A. niger* in the medium at the initial acidic pH values of 2.0 to 4.0, with optimum production at pH 3.0 whereas *P. dierckxii* produced PG in the medium having the initial values ranging from 2.0 to 8.0. Enzyme biosynthesis by *A. niger* seemed to be pH-dependent while that of *P. dierckxii* was independent of the initial pH values of the medium within the range investigated. The initial pH facilitates transport of several species of enzymes across the cell membrane, and thus unfavorable pH of cultivation will limit the growth rate and enzyme production by reducing the accessibility of nutrients (Bajpai 1997). It is unlikely that the initial pH of the culture medium did not affect manganese peroxidase levels produced by *Phanerochaete sorbida*. This may be related to the ability of the fungus to alter the pH of the culture (Carmen 1994). In another study conducted by Krik *et al*. (1978), *Phanerochaete chrysosporium* was employed to produce manganese peroxidase since it has the ability to change the pH of the medium from an initial pH of 4.5-6.0 to around 5.0 after 9 days of cultivation. Amylases can be produced by some fungi at alkaline and acidic pH whereas there are some other fungi which produce amylases within wider pH ranges. Maximum amylase activity by *Aspergillus oryzae* was obtained at pH 7.0-7.5 (Kundu *et al*. 1973). Amylases were produced by *Aspergillus fumigatus* at pH 6.0, *Aspergillus flavus* (pH 8.0) and *Aspergillus niger* at the pH ranges from 3.0 to 7.0 (Absida 1958; Mahmod 1993; Fadel 2000). Further, the optimum initial pH of the medium for amylase production by *Aspergillus ochraceus* was found to be 5.0 although it showed activity at a pH range of 3.0 to 6.0. At pH 6.0 the maximal growth of *A. ochraceus* was 4.5 mg $mL^{-1}$ dry weight (Nahas and Waldemarin 2002). **Table 2** shows the optimum pH on enzymatic fermentation by fungi.

## EFFECT OF MECHANICAL FORCES AND OXYGEN TRANSFER ON ENZYME PRODUCTION CAUSING CHANGES IN MORPHOLOGY OF THE FUNGI

The intensity of agitation affects fungal morphology and oxygen transfer in the bioreactor. In almost all true fungal fermentation, a high agitation rate is essential in order to supply enough mixing and transfer of oxygen, particularly where the fungal cells are being grown in freely dispersed forms that permit to proceed in the non-Newtonian broth. The mechanical forces can cause mycelia to be damaged. Thus, in fungal fermentation, it is necessary to optimize the agitation rate in order to avoid high shear stress on fungal mycelia. Lipase is an enzyme produced by both bacteria and fungi (Vadera and Harmon 1969; Lee and Rhee 1993; Pokorny *et al*. 1994) which has found enormous potentiality in biotechnological processes due to 1) stability in organic solvents, 2) not requiring cofactors, 3) broad specificity of the substrates and 4) showing high enantioselectivity (Jaeger and Reetz 1998).

Submerged fermentation of lipase by fungi depends on agitation and aeration or oxygenation (Frost and Moss 1987). To improve the oxygen transfer rate, perflurocarbon carriers can be used in high oxygen-requiring fermentation processes (Elibol and Mavituna 1996). Perflurocarbons are petroleum-based compounds synthesized by substituting fluorine for the hydrogen molecules of hydrocarbons. They are stable and chemically inert due to the presence of very strong carbon-fluorine bonds. Oxygen solubility in perflurocarbons is 10-20 times higher than that in pure water (Riess and LeBlanc 1982). The influence of oxygen transfer on lipase production by *Rhizopus arrhizus* was carried out under two modes of operation, controlled oxygen concentration and controlled aeration rate. It was found that enzyme production was extensively dependent upon oxygen rather than growth. Oxygen transfer rate was the intrinsic factor determining fungal growth as well as lipase production rather than the concentration of dissolved oxygen. Overall, enzyme productivity depended more strongly on agitation rate than aeration. This could be related to the volumetric oxygen transfer rate, $K_La$ (Murat and Dursun 2000). Li *et al*.

(2002) found a relation between productivity of recombinant enzyme and increase in impeller rate in fed batch fermentation of *Aspergillus oryzae*. The reduced productivity was due to alteration in fungal morphology, increased fragmentation, reduced biomass and undesirable mixing. The impact of agitation on mycellial fragmentation of a recombinant *A. oryzae* which affected protein production was studied by Amanullah *et al*. (1999) in a chemostat culture (5.3 L) where protein production was independent of agitation intensity (550-1100 rpm), despite morphological changes. The study by Li *et al*. was at a large scale (80 $m^3$) in a fed batch system using *A. oryzae* at two different impeller power levels (one 50% higher than the other) and, unlike other reports (Märkl and Bronnenmeier 1985; Smith *et al*. 1990; Makagiansar *et al*. 1993; Johansen *et al*. 1998) increased impeller intensity influenced biomass, fungal morphology or fragmentation behavior very little. Fragmentation dominated growth, branching and clumps were not observed to be abundant as reported by other investigators, where fragmentation dominated fungal growth and most of the biomass existed as small, sparsely branched, free hyphae (Olsvik and Kristiansen 1992a,b; Tucker and Thomas 1992). However, the morphological changes in fungi are largely brought about by the rate of agitation which leads to the formation of a pellet. Higher agitation rate results in smaller and more compact pellets. Joshi *et al*. (1996) claimed that pellets can be broken if their sizes are larger than a critical size and it is brought about as a function of agitation speed. Taguchi *et al*. (1968) and Nielsen *et al*. (1995) reported that there are two mechanisms involved in changing the size of fungal pellets; one is the decrease in diameter by chipping off pellicles from the surface of the pellets and the second one is the direct break up of the pellet structure at higher hydrodynamic loads. However, the study performed by Cui *et al*. (1998) using *Aspergillus awamori* in 2, 15 and 100 L (working volume) stirred tank fermenters does not support the above mechanisms. They concluded that pellet size was influenced by the amount of substrates available for each pellet, the number of the pellets, the agitation rate and the time needed for pellet to grow. For the same number of pellets and agitation rate, a higher concentration of carbohydrate in the medium resulted in a lower number of pellets, a longer growth period and the formation of a larger pellet than when carbohydrate concentration was lower, a larger number of pellets were formed and the growth period was shorter. The size of the pellet was controlled more by growth than by breakage. Changes in fungal morphology can also be associated either with the kind of impeller such as the Rushton turbine (Metz *et al*. 1981; Shamlou *et al*. 1994; Cui *et al*. 1998) or with power input (Smith *et al*. 1990; Makagiansar *et al*. 1993), and, in addition, specific impeller geometrical parameters like the ratios of impeller diameter to vessel diameter (D/T), height of the blade to impeller diameter (W/D), number of blades (n) and the angle of blade (α) can be considered as mechanical damage (Jüsten *et al*. 1996). The intensity of damage caused by various impellers can be measured by image analysis through the hyphal length freely dispersed as well as by the aggregated hyphae. The changes in morphology depend on the impeller geometry. The data obtained with different geometries and various $P/V_L$ levels is correlated on the basis of equal tip speed and two others are based on a combination of the specific energy dissipation rate in the impeller swept volume and the frequency of mycellial circulation through the volume. The function arising from the latter concept is called the energy dissipation/circulation function (EDC). Such correlations were validated through the scale-up experiments performed in mixing vessels of 1.4, 20 and 180 L with a Rushton turbine under fed-batch operated fermentation. The EDC function was found to be a parameter correlated reasonably for hyphal damage over such a range of the scales, while the speed of the tip, $P/V_L$ and specific energy dissipation rate in the impeller region were poor. However, the EDC function can be successfully applied to correlate mycellial fragmentation of *Aspergillus niger* with different agitation intensities and various impellers (Amanullah *et al*. 2000).

## CONCLUDING REMARKS

Fungi are considered to be good sources of enzymes as they can synthesize extracellular (homologous or hetrologous) enzymes for industrial applications. During fermentation processes leading to the formation of enzymes, fungi need carbon, nitrogen, metal ions in macro and micro quantities. They also require oxygen as most of them are aerobic fungi which need oxygen for their growth. The above elements are supplied in the form of nutrients and of course pH and temperature can be adjusted and maintained at the optima level. Oxygen is supplied by gaseous oxygen. The above requirements must be uniformly dispersed throughout the fermenter through proper mechanical mixing and this mechanical mixing can cause damage to the fungal cell. Therefore, to avoid this damage the agitation and kind of agitator must be selected in such a way so as not to influence the fermentation processes thereby decreasing enzyme production.

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