

Optimization and production kinetics for cellulases by wild and mutant strain of *Thermomyces dupontii* in stirred tank reactor

Kinza Nisar¹, Roheena Abdullah^{1*}, Afshan Kaleem¹, Mehwish Iqtedar¹ and Tehreema Iftikhar²

¹Department of Biotechnology, Lahore College for Women University, Lahore, Punjab, Pakistan. ²Department of Botany, Government College University, Katchery Rd, Lahore, 54000, Pakistan. *Author for correspondence. E-mail: roheena_abdullah@yahoo.com

ABSTRACT. The main bottleneck in fermentation technology is scaling up procedure of industrial enzymes according to the biological characteristics of the organism. The current study describes the production kinetics of cellulases in stirred tank reactor by using mutant and wild strains of *T. dupontii*. The fermentation span of both the strains in bioreactor was examined. It was found in mutant strain of *T. dupontii* fermentation time required for optimum production was reduced to 48h as compared to 72h in wild strain. The kinetic studies also exhibited greater value of μ (h^{-1}) in case of mutated strain in comparison with wild strain. The effects of some other critical factors like agitation intensity dissolve oxygen, aeration, temperature, size of inoculum and pH was estimated on enzyme production kinetics. The results shows maximum activity of cellulases was attained at 220 rpm, 15% dissolve oxygen, aeration rate 1.5 vvm, 55°C, 8 % inoculum size and pH 5.5 for both strains respectively. The higher values of enzyme production kinetics i.e. Y_p/x , Q_p , Q_x and q_p in STR in case of mutant strain indicates its superiority over wild strain of *T. dupontii*. Thus mutant thermophilic *T. dupontii* might be a potential candidate for industrial applications.

Keywords: Fermenter; endoglucanase; thermophilic fungi; β -glucosidase; kinetics; wild.

Received on February 3, 2021.

Accepted on February 9, 2021.

Introduction

Thermophilic microorganisms play a vital role in producing industrially important enzymes. These thermophilic microorganisms possess the ability to live in a wide range of temperature from 40°C to 80°C (Jou, Arezi, & Khanahmadi, 2018). Cellulases include a group of hydrolase enzymes, namely: exoglucanases, endoglucanase and β -glucosidase that efficiently degrade the lignocellulolytic biomass in to simple sugars. A wide range of filamentous fungi has been reported to produce cellulases. Some major fungal genera possessing the ability to produce industrial enzyme includes *Penicillium*, *Trichoderma*, *Aspergillus* and *Humicola* etc. (Goyari et al., 2015). Cellulases play an important role in textile, detergents, pharmaceutical, wine, animal feed and paper industries (Taha, Taha, & Faisal, 2015; Imran, Anwar, Zafar, Ali, & Arif, 2018). SmF is a common process and preferred over SSF because of the greater control on different environmental factors such as pH and temperature etc. and easy in handling (Balamurugan & Thirumoorthy, 2018). Generally, bioprocesses are categorized into three main scales, i.e. laboratory scale, pilot plant and production plant (Carvalho et al., 2014). Now a day the most common bioreactor used is stirred tank reactor (STR). The STR is preferred due to their excellent mixing, homogenous system, practically good mass and heat transfer rates, ease in handling and cleaning, better control over some essential factors, such as pH, air supply, temperature, agitation speed and foaming (Socol, Pandey, & Larroche, 2016). The demand of industrial enzymes can only be fulfilled by producing the enzymes at a larger scale. The microbial enzyme production in industry is carried out by a fermentation process that offers several advantages by reducing the cost of the process and utilizing the raw material. In the process of fermentation the pattern of product formation as well as metabolism of each microorganism is based upon the fermentative, physiological, nutritional and genetic nature of that organism.

The exploitation of these microbial metabolisms by controlling the significant fermentation parameters helps us in the economic production of required enzymes at commercial level. Therefore, careful kinetic studies are requisite to monitor the microorganisms' growth at different substrates level and their role in the overall output in the fermentation process. A kinetic study provides prodigious quantitative information about the system behaviour, which is necessary for the study of the fermentation process. The kinetic analysis

also explains the biological significance of each factor and their levels with statistical reliability.

The current work intends to study the impact of time course, aeration rate, agitation speed and dissolved oxygen on the growth kinetics of both wild *T. dupontii* TK-19 and mutant *T. dupontii* EMS 15 in stirred tank reactor.

Material and methods

Microorganism

In the present work, previously isolated wild *T. dupontii* TK-19 and mutant *T. dupontii* EMS 15 were used (Nisar, Abdullah, Kaleem, & Iqtedar, 2020a; Nisar, Abdullah, Kaleem, & Iqtedar, 2020b).

Preparation of vegetative inoculum

The vegetative inoculum was transferred to the production medium at a rate of 8% (v/v) based on total working volume of the fermentation medium. The vegetative inoculum was transferred to the production medium at a rate of 8% (v/v) based on total working volume of the fermentation medium. Inoculum was prepared by adding 10 mL of saline water into 3-4 days old slant containing plentiful fungal growth which was grown at 40°C. The 4% of conidial suspension was transferred in 1000mL of conical flask based on the total working volume of sterilized medium along with 15-20 glass beads. The flasks were incubated for 24h at 40°C in a rotatory shaker (Model: 10X400.XX2.C, SANYO Gallenkamp, PLC, UK) at 200 rpm.

Cellulase production in stirred tank bioreactor

7.5-L stirred tank reactor (Model: Bioflow-110 Fermenter/Bioreactor, USA) with 4.5 L working volume was used for scale up studies. The glass vessel of a bioreactor containing fermentation media of Nisar et al. (2020b) was sterilized in an autoclave (Model: KT-40 L, ALP, Japan) at 121°C for 20 min. Under aseptic conditions the vegetative inoculum was carefully transferred in to the vessel via a port present at the top plate. The temperature of incubation was maintained at 55°C, while agitation and aeration were kept at 200 rpm and 1.0 vvm, respectively. Sterile fermentation medium is subsequently added to the bioreactor while temperature, sterilized air, dissolved oxygen was stabilized and maintained each time through controlled computer system of bioreactor. The 0.1N HCl/NaOH was used in order to maintain the desired pH which was controlled by the system attached to the fermenter. In order to avoid foaming during fermentation process 10% of sterilized silicon oil was used as an antifoaming agent. The fermented broth after a fixed time of incubation was filtered and used for estimation of enzyme.

Estimation of cellulases and Dry cell mass (DCM)

CMCase and FPase activity was determined according to Gao et al. (2008). CMCase activity was estimated by adding 0.5 mL of enzyme along with 0.5 mL of 1% CMC (prepared in 0.1 M citrate buffer pH 5). While in case of FPase 50 mg of Whatman filter paper strips (1×5 cm) was added in 0.5 mL of enzyme along with 0.5mL citrate buffer (0.1 M; pH 5). A blank was also run parallel in which enzyme was substituted by distilled water. The reaction mixture was incubated for 30 min. at 60°C. After, this reducing sugar was measured by Miller (1959) at 546nm. One unit activity of CMcase and FPase was defined as the “amount of enzyme required to release 1 μ mol of glucose from the appropriate substrate per min under standard assay conditions (Shanmugapriya, Saravana, Krishnapriya, Mythili, & Joseph, 2012). While β -glucosidase was determined by following Rajoka & Malik (1997). 0.2 mL of enzyme extract was added in 0.2 mL of p-nitrophenyl- β -D-glucopyranoside (pNPG) and 0.2 mL of acetate buffer (0.2 M; pH 5.0) The reaction mixture was incubated for 10 min. at 45°C. After incubation, 3.0 mL of 1.0 M Na_2CO_3 was added in order to stop the reaction. The blank was run parallel by substituting enzyme with distilled water. The absorbance of the reaction mixture was measured with the help of a spectrophotometer at 400 nm. One unit of enzyme activity was expressed as the quantity of enzyme that corresponded to one micromole of reducing sugars per minute under the standard assay conditions (Ekundayo, Ekundayo, & Ayodele, 2017). Dry cell mass was determined according to Irfan, Javed, and Syed (2011). For this purpose fermented broth was filtered through Whatman filter paper and residue remains on the surface of filter paper washed thrice with tap water in order to remove the fermented broth. After this dry the mycelial mass in preweighed petriplate at 105°C for 2h. The DCM was determined by subtracting the initial weight of petriplate from the final weight. All the experiments were run in triplicates.

Kinetic analysis

Fermentation kinetic parameters were determined by Pirt (1975) and Lawford and Rousseau (1993).

- $Y_{p/x}$ that represents product coefficient yield $U\ g^{-1}$ was found out by the following equation:

$$dp/dx = Y_{p/x} \quad (1)$$

- Q_x (cell mass $g\ L^{-1}\ h^{-1}$) was assessed from maximum slope of cell mass formation verses fermentation time.
- Q_p ($U\ L^{-1}\ h^{-1}$) was calculated from maximum slope of enzyme produced vs fermentation time.
- The specific growth rate μ (h^{-1}) was determined from graph that was plotted between $\ln(x)$ and fermentation time.
- The value of (q_p) specific product yield coefficient ($Ug^{-1}h^{-1}$) was calculated by the following equation:

$$Y_{p/x} \times \mu = q_p \quad (2)$$

Results and discussion

Optimization of different factors for enhanced production of cellulases in STRs

The basic function of the fermenter is to provide a suitable environment in which an organism can grow and efficiently produce a desired product. Kinetic studies play an essential role in the design of bioreactors for enzymes production through fermentation. Mathematical models of bioprocess kinetics aid in data analysis and provide a strategy for solving problems encountered in the design of industrial fermentation processes and bioreactor design. In current project several factors including time of incubation, agitation rate, aeration, oxygen transfer, pH and inoculum size were optimized for scale up studies by thermophilic *T. dupontii*. Similar study has been mentioned by Carvalho et al. (2014) who reported cellulase production in a stirred tank reactor by *Penicillium* sp.

The fermentation process has a significant impact on enzyme productivity. The ideal incubation time for optimum enzyme production differs both with the type of microorganism and fermentation conditions (Ali, Haq, Qadeer, & Iqbal, 2002). On the basis of better cellulase production it was believed that *Penicillium* sp. are considered to be alternate and serious competitors over *Trichoderma* sp. (Singh, Adsul, Vaishnav, Mathur, & Singhania, 2017; Gautam et al., 2011). The rate of fermentation was measured in the case of both wild and mutant *Thermomyces dupontii*. Aseptically aliquots were collected after every eight hours of interval and cellulase productivity was noted up to 96h. Figure 1 showed specific product yield coefficient (q_p) and optimal specific growth rate μ (h^{-1}) for both the strains. The curve showed maximum q_p and μ (h^{-1}) were recorded after 72h for the wild strain however, higher q_p and μ (h^{-1}) were noted after 48h in case of mutant strain. Figure 2 exhibited the differences in enzyme yield and biomass production in case of wild and mutant strain. The kinetic studies, exhibited higher Q_p , Q_x and $Y_{p/x}$ was obtained by mutant *T. dupontii* in comparison to wild *T. dupontii* (Figure 3). The reduction in enzyme activity after the optimum time period might be due to the depletion of essential nutrients, proteolytic digestion or spontaneous enzyme denaturation. Besides this optimal enzyme activity also relies on the type or nature of microbial strain, their genetic makeup as well as its environmental and cultural conditions (Sudan & Bajaj, 2007). Besides this shorter fermentation time in case of mutant thermophilic *T. dupontii* as compared to other thermophilic indicates the strain possess the ability of economic enzyme production Carvalho et al. (2014). The current findings are not in accordance with Carvalho et al. (2014) who stated optimal cellulase production in the case of *Penicillium* at 72 hours.

The mixing efficiency plays an important role in the optimization process of a bioreactor because enzymes are sensitive to mechanical shear stress. Therefore, excessive and lower agitation speed can reduce the efficiency of hydrolysis process (Abd Rahim, Sulaiman, Hamid, Edama, & Baharuddin, 2015). Agitation intensity of the culture broth has multiple effects on microorganisms, such as rupturing of the cell wall, alteration in the fungal morphology, fluctuation in the growth rate and efficiency which in turns affect the rate of desired product formation (Purwanto, Ibrahim, & Sudrajat, 2009). In present study the effect of agitation rate (160-240 rpm) was assessed for cellulase production in STRs (Figure 2b). Maximum enzyme production and cell biomass was recorded at 220 rpm. Reduction in enzyme yield was observed above or below the optimal value by both the thermophilic fungal strains. The comparative analysis of kinetic parameters i.e. Q_p , Q_x and $(Y_{p/x})$ of wild and mutant strain exhibited, higher cellulase activity in mutant strain (Figure 4). The decrease in enzyme productivity might be due to the effect of hydrodynamic stress, which resulted in hyphal disruption and leakage of intracellular compounds. The damaging effect of the shear forces due to increased

intensity of agitation was mentioned by Bakri, Mekaeel, and Koreih (2011). Current findings disagree with Maeda, Barcelos, Santa Anna, and Pereira Jr (2013) who reported 200 rpm for cellulase production.

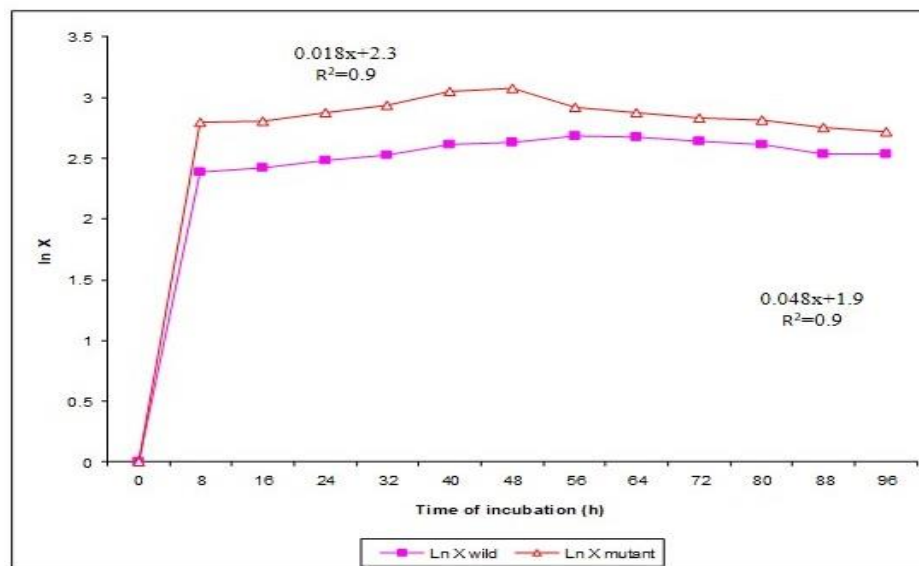


Figure 1. Specific growth rates of both wild and mutant *T. dupontii* with various fermentation time.

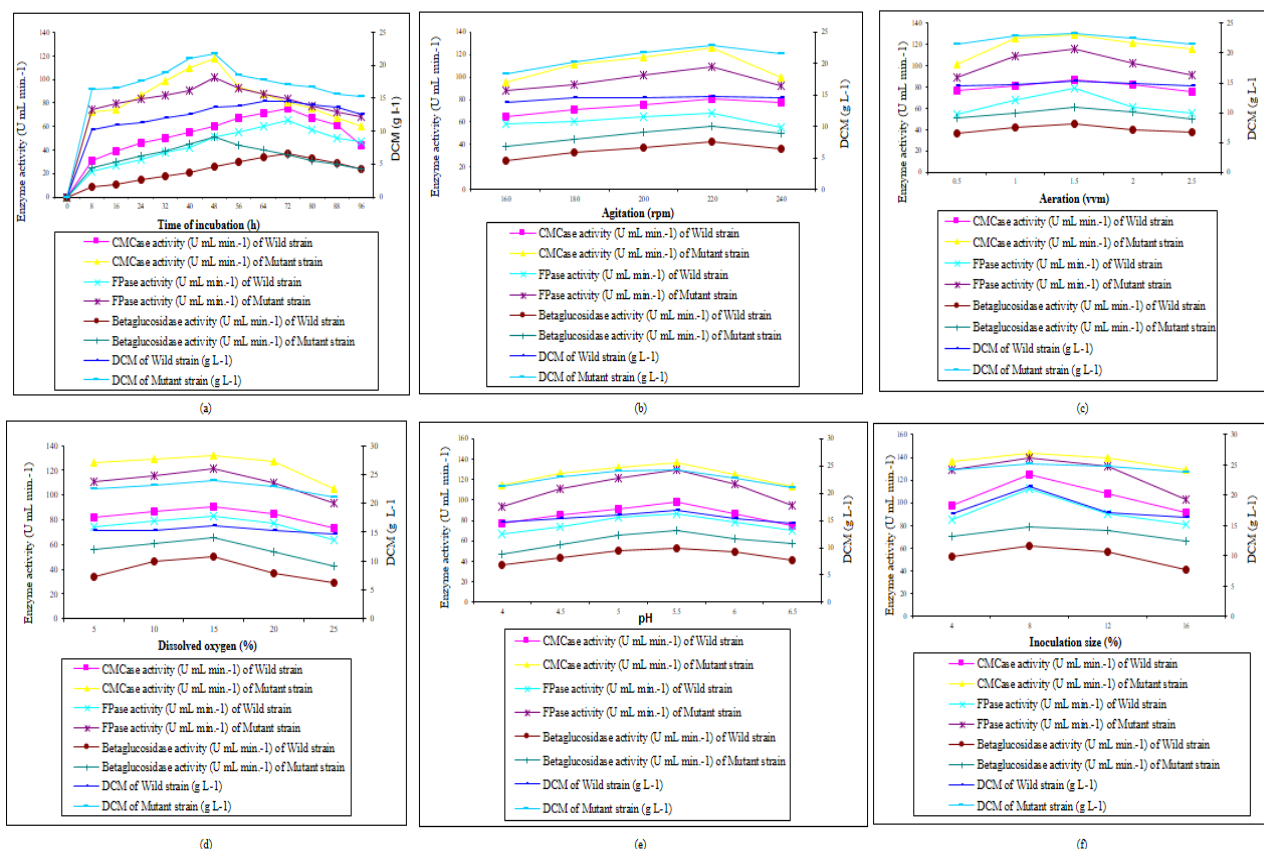


Figure 2. Influence of physical factors on wild and mutant *T. dupontii* (a) Incubation temperature (b) agitation speed (c) aeration rate (d) dissolved oxygen (e) pH (f) inoculum size.

Aeration also determines the oxygenation of the fermentation process. In addition to this it also helps in mixing of the fermentation media, especially in case of low mechanical agitation speed (Zhou et al., 2018). The influence of aeration levels (0.5-2.5 vvm) was also tested for cellulases production. Figure 2c showed that 1.5 vvm aeration rate supports the optimal enzyme yield and biomass production for both the strains. However, beyond the ideal level, drop in cell biomass and enzyme production was recorded. The kinetics

studies show the higher values of $Y_{p/x}$, Q_p and Q_x for mutant *Thermomyces dupontii* TK-EMS15 than wild *Thermomyces dupontii* TK-19 (Figure 5). Cellulases production gradually reduced when the aeration level was increased from 1.5 vvm. However, inactivation of enzyme in highly aerated culture is might be due to irreversible oxidation of amino acid residues of the enzyme structure (Bakri et al., 2011). Similar findings were described by Kim et al. (2011) who reported maximal cellulase productivity at 1.5vvm aeration level.

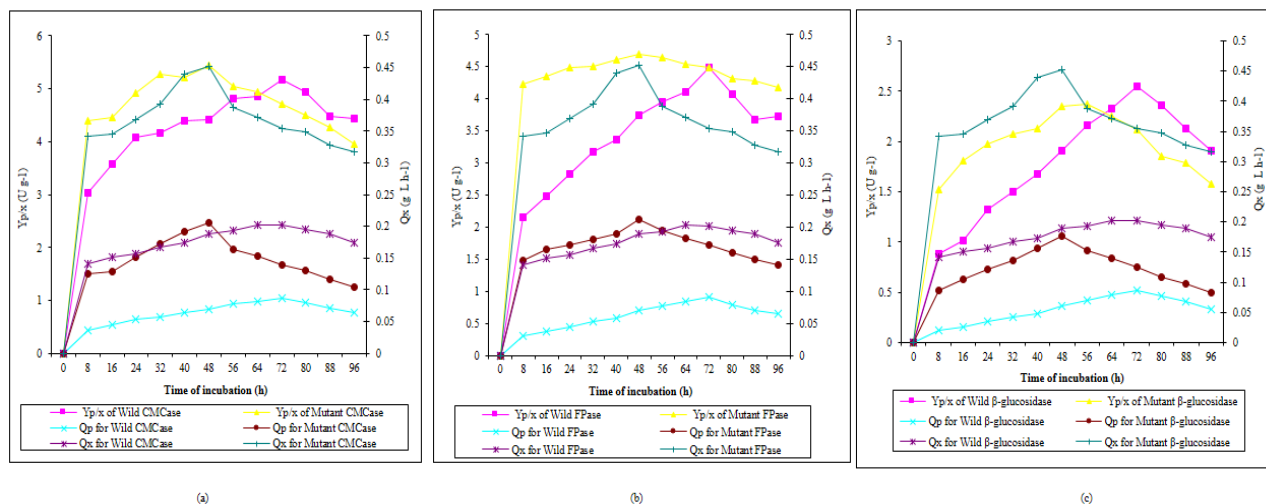


Figure 3a-c. Impact of various incubation time on Q_p , Q_x and $Y_{p/x}$ of *T. dupontii* on the production of cellulases.

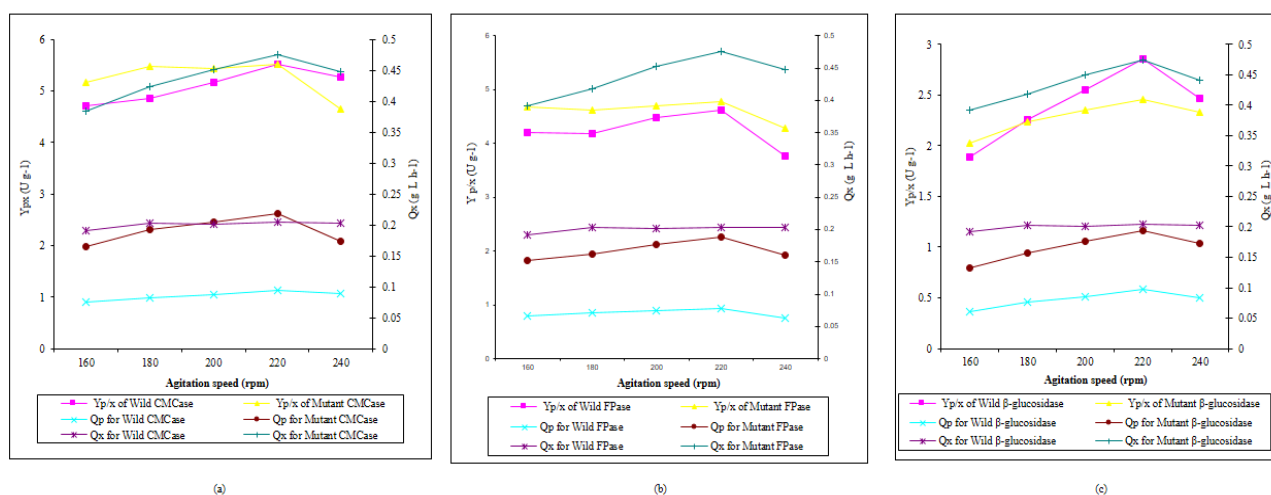


Figure 4a-c. Impact of various agitation speeds on Q_p , Q_x and $Y_{p/x}$ of *T. dupontii* on the production of cellulases.

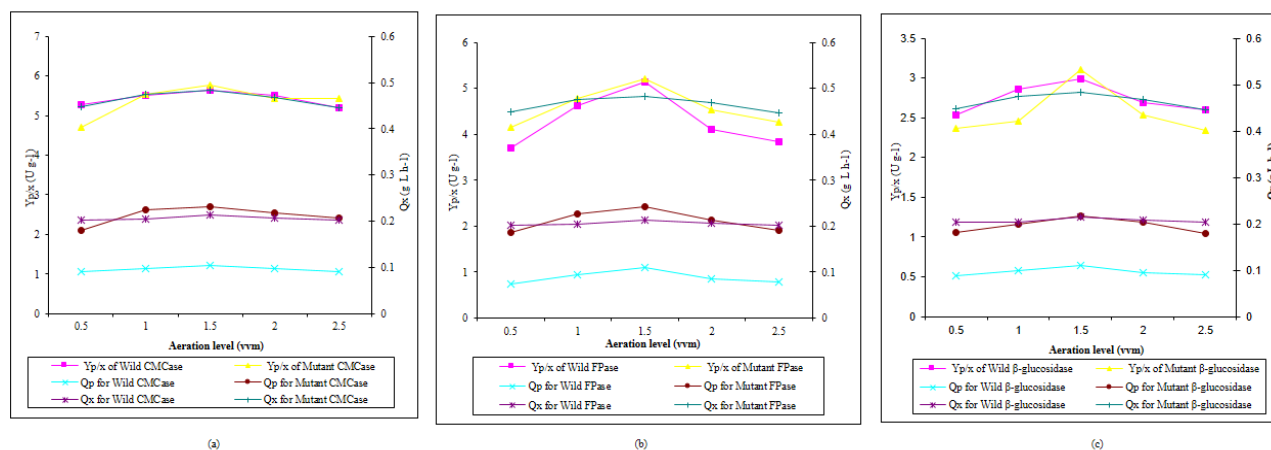


Figure 5a-c. Impact of various levels of aeration on Q_p , Q_x and $Y_{p/x}$ of *T. dupontii* on the production of cellulases.

The continuous supply of dissolved oxygen to the organism is needed for the aerobic fermentation. The *Thermomyces dupontii* is an aerobic filamentous fungus, so dissolved oxygen concentration might have a high influence on enzyme production kinetics. In submerged fermentation, by increasing the speed of agitation the concentration of dissolved oxygen can be increased. However, filamentous fungi have propensity to agglomerate and form clumps at high turbulences in liquid medium, which lowers the interaction of hyphal surface with oxygen and nutrients that in turn might lead to lesser enzyme productivity (Vintila, Croitoriu, Dragomirescu, & Nica, 2010). In the current investigation the impact of different dissolved oxygen levels ranging from 5-20% were also tested (Figure 2d). In case of wild and mutant strains, maximal cellulase production was recorded in 15% of dissolved oxygen level. Above or below the maximal level, reduction in biomass and enzyme production was noted. Fermenter kinetics studies i.e. Yp/x, Qx and Qp also showed the superiority of mutant strain over the parental *T. dupontii* (Figure 6). High or low concentrations of dissolved oxygen might have negative effect on enzyme production and can reduce the growth of the microorganisms. At higher concentration, oxygen might cross from its saturation level which in turn produces some of the toxic compounds. However, at lower concentration the air supply may cause anaerobic condition which retard the fungal growth and consequently enzyme production (Zafar, Aftab, Iqbal, Ud Din, & Saleem, 2019).

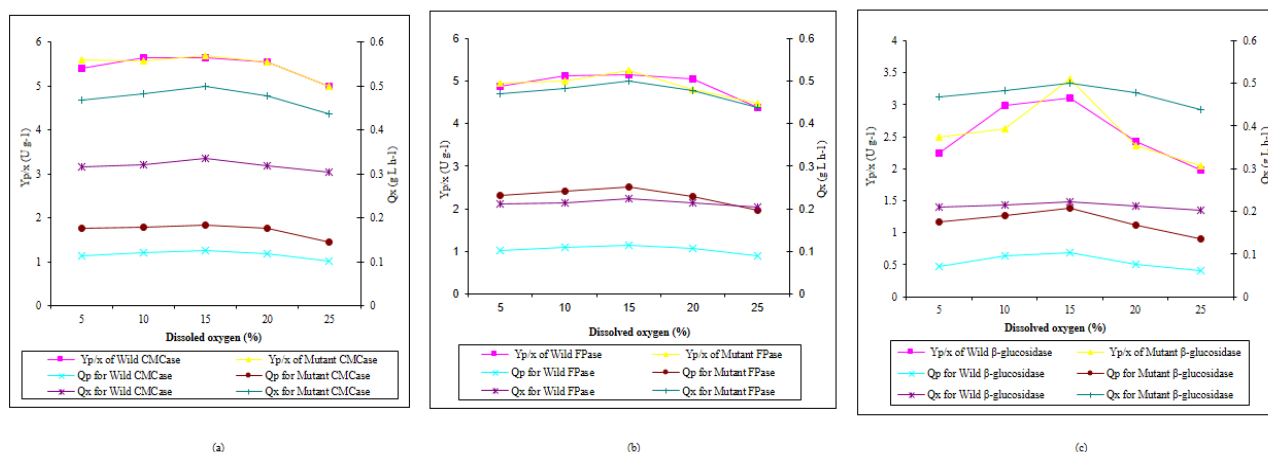


Figure 6a-c. Impact of different level of dissolved oxygen on Qp, Qx and Yp/x of *T. dupontii* on the production of cellulases.

Among all the physical factors, the pH of the growth medium plays a significant role by inducing morphological changes in fungi as well as enzyme secretion (Pradeep & Narasimha, 2011). Different initial pH (4-6.5) was evaluated for cellulase production in STRs. In present investigation, pH 5.5 was found to be best for optimum enzyme production in case of both strains (Figure 2e). Higher titers of Qx, Qp and Yp/x was observed in the mutant strain as compared to the parental strain (Figure 7). Decreasing trend in enzyme production was found with the escalation in pH value. The decline in productivity with further rise in pH value can be explained by the reason that enzymes are sensitive to pH and minor change in pH might cause a drop in enzyme production (Nguyen, Kumla, Suwannarach, Penkhruue, & Lumyong, 2019).

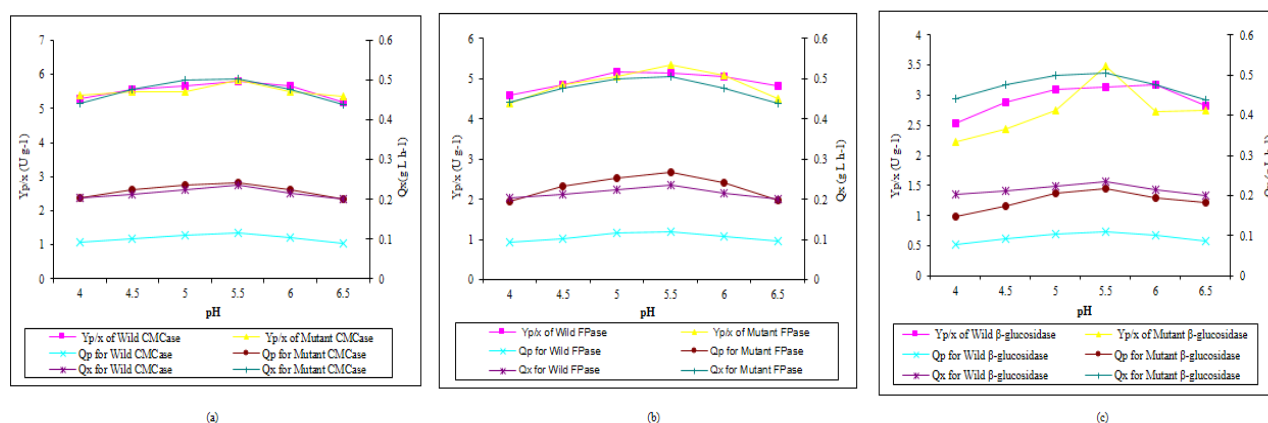


Figure 7a-c. Effect of various initial pH on Qp, Qx and Yp/x of *T. dupontii* on the production of cellulases.

A lower inoculum size needs a longer time period for fungal colonies to multiply at a certain number in order to produce enzyme by utilizing the substrate in the medium. However, large inoculum size would ensure the rapid proliferation and biomass synthesis. For optimum enzyme production, it is necessary to find the balance between the accessible nutrients and increasing biomass production. Higher inoculum size might expedite the rate of fungal growth but on the other hand also accelerate nutrient depletion rate. The exhaustion of essential nutrient retards the fungal growth and, consequently yield of enzyme production (Kumaran, Sastry, & Vikineswary, 1997). Influence of various inoculum sizes ranging from 4-16% (v v⁻¹) were studied in case of both wild and mutant strains. Figure 2f depicts that maximal cellulase production was achieved at 8% of inoculum size (2 x10⁷ conidia ml⁻¹). The higher values of kinetic parameters such as Yp/x and volumetric rates i.e. (Qp, Qx) was obtained for mutant strain than wild strain (Figure 8). Cell biomass and enzyme productivity decreases with further increase in inoculum size which may be due to the quick synthesis of fungal biomass at the certain limit which in turn causes the unavailability of essential nutrients (Prakasham, Rao, & Sarma, 2006; Khattab, Azzaz, El Tawab, & Murad, 2019). The present results are differing to Khattab et al. (2019) who optimized 4 % inoculum size for the production of cellulases.

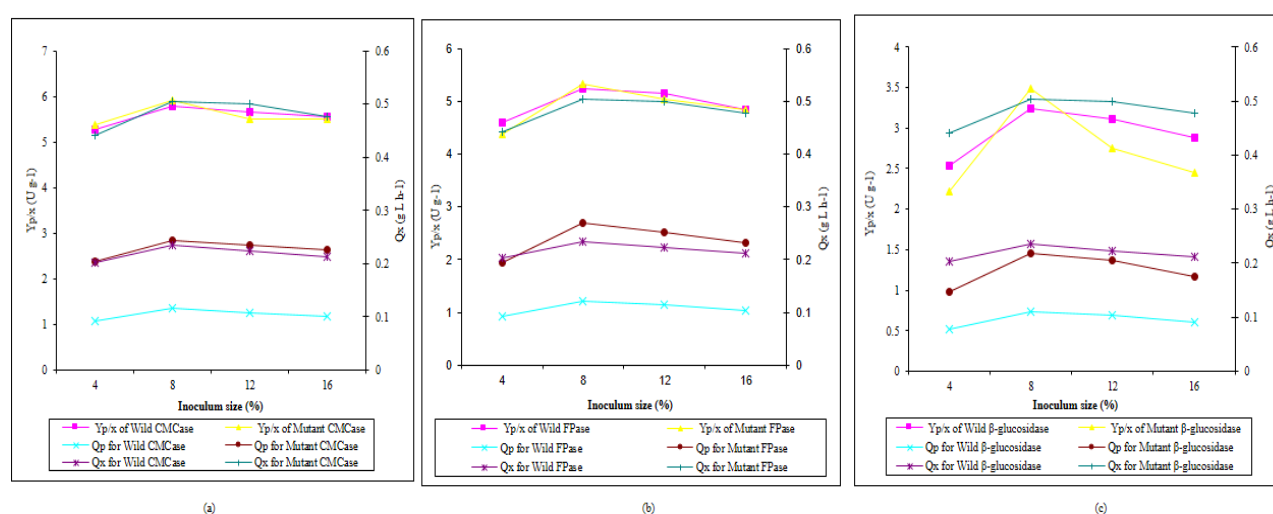


Figure 8a-c. Impact of different inoculum size on Qp, Qx and Yp/x of *T. dupontii* on the production of cellulases.

Conclusion

Kinetic studies in present work provided sufficient insight on cellulase production by thermophilic mutant *Thermomyces dupontii*-EMS15. The maximum activity of cellulases was attained at 220 rpm, 15% dissolve oxygen, aeration rate 1.5 vvm, 55°C, 8 % inoculum size and pH 5.5 for both strains respectively. It was concluded that *T. dupontii* was considered to be the most efficient producer for cellulases at industrial scale.

References

- Abd Rahim, S. N., Sulaiman, A., Hamid, K. H. K., Edama, N. A., & Baharuddin, A. S. (2015). Effect of agitation speed for enzymatic hydrolysis of tapioca slurry using encapsulated enzymes in an enzyme bioreactor. *International Journal of Chemical Engineering and Applications*, 6(1), 38-41.
DOI: <http://dx.doi.org/10.7763/IJCEA.2015.V6.447>
- Ali, S., Haq, I. U., Qadeer, M. A., & Iqbal, J. (2002). Production of citric acid by *Aspergillus niger* using cane molasses in a stirred fermentor. *Electronic Journal of Biotechnology*, 5(3), 19-20.
DOI: <http://doi.org/10.2225/vol5-issue-fulltext-3>
- Bakri, Y., Mekaeel, A., & Koreih, A. (2011). Influence of agitation speeds and aeration rates on the xylanase activity of *Aspergillus niger* SS7. *Brazilian Archives of Biology and Technology*, 54(4), 659-664.
DOI: <http://dx.doi.org/10.1590/S1516-89132011000400003>
- Balamurugan, V. B., & Thirumoorthy, G. T. (2018). Production of cellulase by filamentous fungi with sorghum as substrates. *International Journal of Research in Engineering, Science and Management*, 1(1-8), 13-17.

- Carvalho, M. L. d. A. d., Carvalho, D. F., Gomes, E. d. B., Maeda, R. N., Santa Anna, L. M. M., Castro, A. M. d., & Pereira Jr., N. (2014). Optimization of cellulase production by *Penicillium funiculosum* in a stirred tank bioreactor using multivariate response surface analysis. *Enzyme Research* 2014(703291), 1-8. DOI: <https://doi.org/10.1155/2014/703291>
- Ekundayo, F. O., Ekundayo, E. A., & Ayodele, B. B. (2017). Comparative studies on glucanases and β -glucosidase activities of *Pleurotus ostreatus* and *P. pulmonarius* in solid state fermentation. *Mycosphere*, 8(8), 1201-1209. DOI: <http://doi.org/10.5943/mycosphere/8/8/16>
- Gao, J., Weng, H., Zhu, D., Yuan, M., Guan, F., & Xi, Y. (2008). Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. *Bioresource Technology*, 99(16), 7623-7629. DOI: <https://doi.org/10.1016/j.biortech.2008.02.005>
- Gautam, S. P., Bundela, P. S., Pandey, A. K., Khan, J., Awasthi, M. K., & Sarsaiya, S. (2011). Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. *Biotechnology Research International*, 2011(810425), 1-8. DOI: <https://doi.org/10.4061/2011/810425>
- Goyari, S., Devi, S. H., Bengyella, L., Khan, M., Sharma, C. K., Kalita, M. C., & Talukdar, N. C. (2015). Unveiling the optimal parameters for cellulolytic characteristics of *Talaromyces verruculosus* SGMNPF3 and its secretory enzymes. *Journal of Applied Microbiology*, 119(1), 88-98. DOI: <http://doi.org/10.1111/jam.12816>
- Imran, M., Anwar, Z., Zafar, M., Ali, A., & Arif, M. (2018). Production and characterization of commercial cellulase produced through *Aspergillus Niger* IMMIS1 after screening fungal species. *Pakistan Journal of Botany*, 50(4), 1563-1570.
- Irfan, M., Javed, J., & Syed, Q. (2011). UV mutagenesis of *Aspergillus niger* for enzyme production in submerged fermentation. *Pakistan Journal of Biochemistry and Molecular Biology* 44(4), 137-140.
- Jou, A. D., Arezi, I., & Khanahmadi, M. (2018). Scale-up thermostable α -amylase production in lab-scale fermenter using rice husk as an elicitor by *Bacillus licheniformis*-AZ2 isolated from Qinarje Hot Spring (Ardebil Prov. of Iran). *Periodicum Biologorum*, 120(1), 11-21. DOI: <http://doi.org/10.18054/pb.v120i1.6775>
- Khattab, M. S. A., Azzaz, H. H., El Tawab, A. M. A., & Murad, H. A. (2019). Production optimization of fungal cellulase and its impact on ruminal degradability and fermentation of diet. *International Journal of Dairy Science*, 14(2), 61-68. DOI: <http://doi.org/10.3923/ijds.2019.61.68>
- Kim, H.-J., Lee, Y.-J., Gao, W., Chung, C.-H., Son, C.-W., & Lee, J.-W. (2011). Statistical optimization of fermentation conditions and comparison of their influences on production of cellulases by a psychrophilic marine bacterium, *Psychrobacter aquimaris* LBH-10 using orthogonal array method. *Biotechnology and Bioprocess Engineering*, 16(3), 542-548. DOI: <http://doi.org/10.1007/s12257-010-0457-5>
- Kumaran, S., Sastry, C. A., & Vikineswary, S. (1997). Laccase, cellulase and xylanase activities during growth of *Pleurotus sajor-caju* on sago hampas. *World Journal of Microbiology and Biotechnology*, 13(1), 43-49. DOI: <http://doi.org/10.1007/BF02770806>
- Lawford, H. G., & Rousseau, J. D. (1993). Effects of pH and acetic acid on glucose and xylose metabolism by a genetically engineered ethanologenic *Escherichia coli*. *Applied Biochemistry and Biotechnology*, 39(1), 301-322. DOI: <http://doi.org/10.1007/BF02918999>
- Maeda, R. N., Barcelos, C. A., Santa Anna, L. M. M., & Pereira Jr, N. (2013). Cellulase production by *Penicillium funiculosum* and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. *Journal of Biotechnology*, 163(1), 38-44. DOI: <https://doi.org/10.1016/j.jbiotec.2012.10.014>
- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31(3), 426-428. DOI: <https://doi.org/10.1021/ac60147a030>
- Nguyen, K. A., Kumla, J., Suwannarach, N., Penkhrue, W., & Lumyong, S. (2019). Optimization of high endoglucanase yields production from polypore fungus, *Microporus xanthopus* strain KA038 under solid-state fermentation using green tea waste. *Biology Open*, 8(11), bio047183. DOI: <http://doi.org/10.1242/bio.047183>

- Nisar, K., Abdullah, R., Kaleem, A., & Iqtedar, M. (2020b). Application of response surface methodology for statistical optimization of carboxymethylcellulase by *Thermomyces dupontii* TK-19 using submerged fermentation. *Revista Mexicana de Ingeniería Química*, 19(2), 903-911. DOI: <http://doi.org/10.24275/rmiq/Bio895>
- Nisar, K., Abdullah, R., Kaleem, A., Iqtedar, M., & Saleem, F. (2020a). Hyper production of carboxy methyl cellulase by *Thermomyces dupontii* utilizing physical and chemical mutagenesis. *Revista Mexicana de Ingeniería Química*, 19(2), 617-625. DOI: <http://doi.org/10.24275/rmiq/bio823>
- Pirt, S. J. (1975). *Principles of microbe and cell cultivation*. Oxford, UK: Blackwell Scientific Publications.
- Pradeep, M. R., & Narasimha, G. (2011). Production of cellulase enzyme by mutant fungal strain *Aspergillus niger* in submerged fermentation. *BioTechnology – An India Journal*, 5(3), 148-152.
- Prakasham, R. S., Rao, C. S., & Sarma, P. N. (2006). Green gram husk—an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation. *BioResource Technology*, 97(13), 1449-1454. DOI: <https://doi.org/10.1016/j.biortech.2005.07.015>
- Purwanto, L. A., Ibrahim, D., & Sudrajat, H. (2009). Effect of agitation speed on morphological changes in *Aspergillus niger* hyphae during production of tannase. *World Journal of Chemistry*, 4(1), 34-38.
- Rajoka, M. I., & Malik, K. A. (1997). Cellulase production by *Cellulomonas biazotea* cultured in media containing different cellulosic substrates. *BioResource Technology*, 59(1), 21-27. DOI: [https://doi.org/10.1016/S0960-8524\(96\)00136-8](https://doi.org/10.1016/S0960-8524(96)00136-8)
- Shanmugapriya, K., Saravana, P. S., Krishnapriya, M. M., Mythili, A., & Joseph, S. (2012). Isolation, screening and partial purification of cellulase from cellulase producing bacteria. *International Journal of Advanced Biotechnology and Research*, 3(1), 509-514.
- Singh, A., Adsul, M., Vaishnav, N., Mathur, A., & Singhania, R. R. (2017). Improved cellulase production by *Penicillium janthinellum* mutant. *Indian Journal of Experimental Biology*, 55, 436-440.
- Soccol, C. R., Pandey, A., & Larroche, C. (Eds.). (2013). *Fermentation Processes Engineering in the Food Industry*. Boca Raton: FL: CRC Press.
- Sudan, R., & Bajaj, B. K. (2007). Production and biochemical characterization of xylanase from an alkali-tolerant novel species *Aspergillus niveus* RS2. *World Journal of Microbiology and Biotechnology*, 23, 491-500. DOI: <http://doi.org/10.1007/s11274-006-9251-0>
- Taha, A. S. J., Taha, A. J., & Faisal, Z. G. (2015). Purification and kinetic study on cellulase produced by local *Trichoderma viride*. *Journal of Nature and Science*, 13, 87-90.
- Vintila, T., Croitoriu, V., Dragomirescu, M., & Nica, D. (2010). The effects of bioprocess parameters on cellulase production with *Trichoderma viride* CMIT35. *Scientific Papers: Animal Science and Biotechnologies*, 43(1), 337-340.
- Zafar, A., Aftab, M. N., Iqbal, I., Ud Din, Z., & Saleem, M. A. (2019). Pilot-scale production of a highly thermostable α -amylase enzyme from *Thermotoga petrophila* cloned into *E. coli* and its application as a desizer in textile industry. *RSC Advances*, 9(2), 984-992. DOI: <https://doi.org/10.1039/C8RA06554C>
- Zhou, Y., Han, L.-R., He, H.-W., Sang, B., Yu, D.-L., Feng, J.-T., & Zhang, X. (2018). Effects of agitation, aeration and temperature on production of a novel glycoprotein GP-1 by *Streptomyces kanasensis* ZX01 and scale-up based on volumetric oxygen transfer coefficient. *Molecules*, 23(1), 125. DOI: <https://doi.org/10.3390/molecules23010125>